PREVALENCE OF LEPTOSPIROSIS – A STUDY AMONG CANINES AND CANINE PET OWNERS AND OTHER OCCUPATIONAL RISK GROUPS

Dissertation submitted in partial fulfillment of the
Requirement for the award of the Degree of
M.D. MICROBIOLOGY (BRANCH IV)

CHENNAI MEDICAL COLLEGE HOSPITAL AND RESEARCH CENTRE
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CHENNAI, TAMILNADU

APRIL– 2016

CERTIFICATE

This is to certify that the dissertation entitled, **"Prevalence of Leptospirosis – A study among canines and canine pet owners and other occupational risk groups"** by **Dr.J. Meera,** Post graduate in Microbiology (2013-2016), is a bonafide research work carried out under our direct supervision and guidance and is submitted to The Tamilnadu Dr. M.G.R. Medical University, Chennai, for M.D. Degree Examination in Microbiology, Branch IV, to be held in **April 2016.**

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DECLARATION

I solemnly declare that the dissertation titled " **Prevalence of Leptospirosis- A study among canines and canine pet owners and other occupational risk groups** " is done by me at Chennai Medical College Hospital and Research centre, Trichy.

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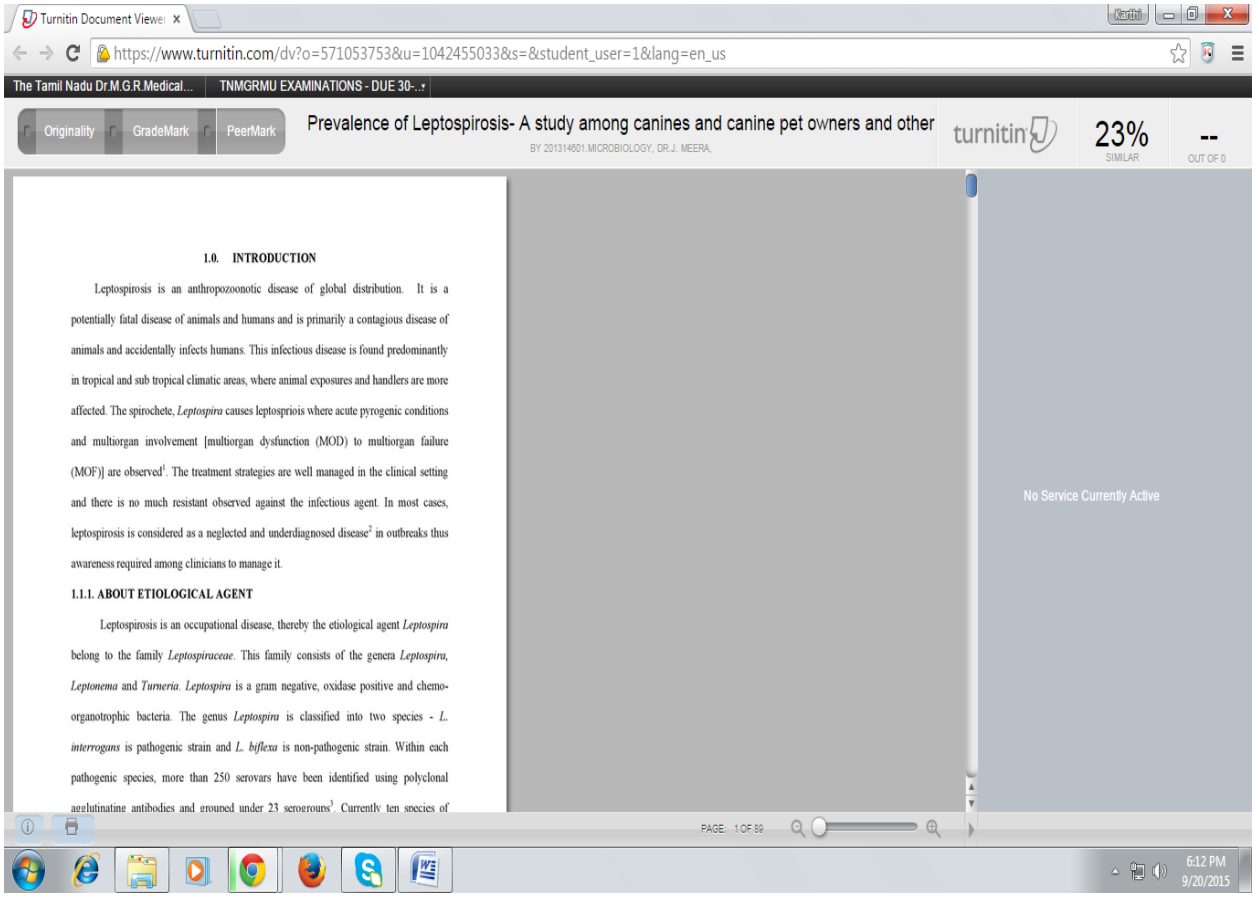
To whomsoever it may Concern.

This is to make it known to all that the study undertaken by **Dr.J.Meera**, a post graduate student in Microbiology at the Chennai Medical College Hospital and research centre, Irungalur on the Leptospira species in pet dogs is in no way harmful to the animals.

She intends to draw only an admissible quantity of blood from the dogs and we have absolutely no objection on the method of drawal and assay procedures to be carried out in this study.

President.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1.0. INTRODUCTION
Leptospirosis is an anthropozoonotic disease of global distribution. It is a potentially fatal disease of animals and humans and is primarily a contagious disease of animals and accidentally infects humans. This infectious disease is found predominantly in tropical and sub tropical climatic areas, where animal exposures and handlers are more affected. The spirochete, Leptospira causes leptospriois where acute pyrogenic conditions and multiorgan involvement [multiorgan dysfunction (MOD) to multiorgan failure (MOF)] are observed[1]. The treatment strategies are well managed in the clinical setting and there is no much resistant observed against the infectious agent. In most cases, leptospirosis is considered as a neglected and underdiagnosed disease[2] in outbreaks thus awareness required among clinicians to manage it.
1.1.1. ABOUT ETIOLOGICAL AGENT
Leptospirosis is an occupational disease, thereby the etiological agent Leptospira belong to the family Leptospiraceae. This family consists of the genera Leptospira, Leptonema and Turneria. Leptospira is a gram negative, oxidase positive and chemo-organotrophic bacteria. The genus Leptospira is classified into two species - L. interrogans is pathogenic strain and L. biflexa is non-pathogenic strain. Within each pathogenic species, more than 250 serovars have been identified using polyclonal agglutinating antibodies and grouped under 23 serogroups[3]. Currently ten species of
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ABBREVIATIONS

MOD -	Multi Organ Dysfunction
MOF -	Multi Organ Failure
MAT -	Microscopic Agglutination Test
CAAT -	Cross Absorption Agglutination Test
ELISA -	Enzyme Linked Immuno Sorbent Assay
EMJH -	*E*llinghausen, *M*cCullough, *J*ohnson and *H*arris
LERG -	*Leptospirosis burden Epidemiology Reference Group*

ABSTRACT

Introduction:

Leptospirosis is a worldwide zoonotic disease. Leptospirosis is an anthropozoonotic disease of global distribution. It is a potentially fatal disease of animals and humans and is primarily a contagious disease of animals and accidentally infects humans. This infectious disease is found predominantly in tropical and sub tropical climatic areas, where animal exposures and handlers are more affected. The spirochete, *Leptospira* causes leptospriois where acute pyrogenic conditions and multiorgan involvement multiorgan dysfunction (MOD) to multiorgan failure (MOF)] are observed.

Materials and Methods:

A prospective cross-sectional observational study was undertaken between April 2014 to March 2015 to identify the presence of Leptospiral antibodies in 142 subjects (95 males and 77 females), in and around Thiruchirapalli, Tamilnadu, INDIA. Fifty three dogs of age 2 months to 15 years were included in the study to screen for the presence of leptospires and its specific antibodies. Enzyme Linked Immuno Sorbent Assay using IgM *Leptospira* ELISA Panbio kit and Microscopic Agglutination Test (MAT) was performed for all human subjects. For canine samples, blood culture with EMJH media was done along with MAT. Positive blood cultures were sent to reference laborartory in Port Blair, Andhaman & Nicobar, India for obtaining Cross Absorption Agglutination Test (CAAT) reports.

Results:

The seroprevalence of the asymptomatic study subjects in this study were 9.8%. The predominant serovar was Australis in both pet owner and butcher group and Canicola in farmers.

In Cross Absorption Agglutination Test (CAAT) for blood culture positive (24.5%) pet dogs' samples, the serogroup Javanica dominated followed by Canicola, Grippotyphosa and Pomona. This study shows the prevalence of leptospires and its antibodies in unvaccinated and irregularly vaccinated pet dogs in comparison to regularly vaccinated pet dogs in Thiruchirapalli, Tamil Nadu, INDIA.

Overall seropositivity for human subjects by ELISA was 4.6% (8/172) and MAT reactivity was 9.8% (17/172).

Conclusion:

The present study emphasizes the possible role of transmission of Leptospirosis from the canine pets to their owners as, 60% correlation observed between pet owners' and their pet dogs based on serovar.

Key words: *Leptospira*, ELISA, MAT, CAAT, Seroprevalence.

1.0. INTRODUCTION

Leptospirosis is an anthropozoonotic disease of global distribution. It is a potentially fatal disease of animals and humans and is primarily a contagious disease of animals and accidentally infects humans. This infectious disease is found predominantly in tropical and sub tropical climatic areas, where animal exposures and handlers are more affected. The spirochete, *Leptospira* causes leptospriois where acute pyrogenic conditions and multiorgan involvement [multiorgan dysfunction (MOD) to multiorgan failure (MOF)] are observed[1]. The treatment strategies are well managed in the clinical setting and there is no much resistant observed against the infectious agent. In most cases, leptospirosis is considered as a neglected and underdiagnosed disease[2] in outbreaks thus awareness required among clinicians to manage it.

1.1.1. ABOUT ETIOLOGICAL AGENT

Leptospirosis is an occupational disease, thereby the etiological agent *Leptospira* belong to the family *Leptospiraceae*. This family consists of the genera *Leptospira*, *Leptonema* and *Turneria*. *Leptospira* is a gram negative, oxidase positive and chemo-organotrophic bacteria. The genus *Leptospira* is classified into two species - *L. interrogans* is pathogenic strain and *L. biflexa* is non-pathogenic strain. Within each pathogenic species, more than 250 serovars have been identified using polyclonal agglutinating antibodies and grouped under 23 serogroups[3]. Currently ten species of leptospires are described in this pathogenic genus including alexanderi, borgpetersenii, fainei, inadai, interrogans, krishneri, meyeri, noguchii, santarosai and weilii. Three

species belong to non pathogenic saprophytic group are biflexa, hollandia and wolbachia. Among non pathogens, there are 65 serovars grouped under 38 serogroups[4]. Pathogenic leptospires are important for their ability to cause infection in animals and further cause disease in humans. Non pathogenic leptospires need to be differentiated from pathogens to avoid confusion in diagnosis and epidemiology.

1.1.2. HABITAT

Leptospires are highly motile with cork screw movement with endoflagellation and that are freely living in surface waters, sewage effluents, soil and mud. They feed by surface adhesion where long chain fatty acids may helpful[5]. Pathogenic leptospires adapt to the environmental conditions including salinity and temperature of febrile mammals mainly on rodents, renal tubular and bladder urine, soil and surface water [6]. This organism is found in both urban and rural environmental conditions [7 8 9].

1.1.3. MORPHOLOGY AND CULTURAL CHARACTERIZATION

The size of the leptospires is about 10-20 micron long where the flagella originate in a disc rotor and hooked proximal end structure in the cell wall and translationally motile and spin fast. The both ends of this organism are bent or hooked, but straight forms also occur while rotate and travel more slowly than hooked forms. Due to this phenomenon, the leptospires are visualized by dark-field illumination or phase contrast microscopy[4]. This spirochete is an obligate aerobes and cultivable in a suitable aerated medium at 30ºC and an optimal pH of 7.2 to 7.6[10]. The medium of choice for leptospiral growth is Ellinghausen, MacCulough, Jansen and Harris semi solid medium supplemented with bovine serum albumin, tween 80, vitamin solutions. A selective agent

5 Fluorouracil (5FU) is quite useful to eliminate other contaminants[11]. The mineral salts including calcium chloride, zinc sulphate, copper sulphate, magnesium chloride and sodium pyruvate are defined as enhancing factors for leptospiral growth. Due to the mineral overflow and disintegration, leptospires are resistant to 5-fluorouracil[4]. Liquid media are much useful in diagnosis especially for microscopic agglutination test (MAT) and antileptosprial activity. Growth is visualized in semisolid medium as Dinger's ring and it is defined as the white ring decending half inch down from the surface of the medium as the days passes and referred as "concentration dependent"; lack of rings does not mean an absence of leptospires[12 13].

1.2. MODE OF TRANSMISSION

The main reservoirs of the agent are wild and domestic animals. It is transmitted through minute abrasion in skin and mucous membrane and indirectly through contaminated water while swimming. It can also spread from moist soil and vegetation infected by diseased animal urine. Spread of infection can also occur through contaminated food and through infected droplets. The incubation period is 10 days (2 to 30 days). Among the animals, rodents (permanent carrier), and others (temporary carrier) like dogs, horses, cattles, swine, cat act as reservoir to transmit infection to humans[14 15]. Mostly this spirochetal infection occurs during the monsoon months (September to January) every year. These organisms can survive for 6 hours in dry soil and 6 months in flooded condition further enter into the host. The occupational risk groups are farmers, municipal workers, rice mill workers, buchers, militarians, swimmers, miner workers, veterinarians, laboratory people who are handling live leptospires, pet animal carers,

cattle handlers etc. This infection is prone to all ages and races who are susceptible, adult men are more frequently infected because they tend to work in high-risk jobs[16 17]. The number of cases in a region often fluctuates from year to year due to various factors such as rainfall, flooding and animal infections[18].

1.3. PATHOGENESIS

Leptospires enter the human system via small abrasions or other breaches of the surface integument. They may also enter directly into the bloodstream or lymphatic system via the conjunctiva, the genital tract in some animals, the nasopharyngeal mucosa, possibly through a cribriform plate, rare cases of the lungs following inhalation of aerosols, or through an invasion of the placenta from the mother to the foetus at any stage of pregnancy in mammals[4 10]. Drinking or inhalation of contaminated water following immersion can also cause leptospirosis. The primary lesion in leptospirosis is disruption of the integrity of the cell membrane of the endothelial cells lining small blood vessels leads to capillary leakage and hemorrhages. Widespread petechial hemorrhages appear in all organs and tissues, especially the lungs, omentum and pericardium. The resulting anatomical damage causes liver involvement inlduing chronic jaundice, hepatomegaly and renal failure that can be fatal. Leptospires enter the cerebrospinal fluid (CSF) in the early septicemia phase of the illness, but there is little evidence of inflammatory response in the CSF[19].

The ocular involvement leads to invasion of leptospires during acute infection, but they are trapped there and cannot move out after the local vasodilation and inflammation subside. Antibodies from circulation can enter and cause an acute hypersensitivity

uveitis. Leptospires are able to persist in some anatomically localized and immunological sites, after antibodies and phagocytes have cleaved leptospires from all other sites. However, humans do not remain carriers for long and the urine is free of leptospires at the time of clinical recovery[4 20].

1.4. CLINICAL FEATURES

An acute febrile illness with headache, myalgia especially of calf muscle, arthralgia, and prostration associated with jaundice, anuria/ oliguria, conjunctival suffusion, haemorrhages, meningeal irritation, cardiac arrythemia, renal involvement, skin rashes may be considered and included for the diagnosis of leptospirosis[4 21]. The natural course of leptospirosis comprises of two distinct clinical phases including septicemic and immune. Humans typically become ill 7 to 12 days after exposure to leptospires.

Myalgias classically involving the paraspinal, calf and abdominal muscles and conjunctival suffusion and a nonspecific febrile illness raise suspicion for diagnosis[22].

As a result of the body's immunologic response by producing immunoglobulin M antibodies immune phase results and can last longer than one month[23].

Varying degrees of jaundice, pancreatitis, hepatomegaly and myocarditis can also occur. Observation of thrombocytopenia also detected but need to differentiate with other infectious diseases induced thrombocytopenia[24 25].

A special condition of leptospirosis is Weil's disease, which is a most severe form of the disease. Weil's disease can occur at the end of the first stage and peaks during the

second stage but can occur at any time during acute leptospirosis as a single, progressive illness where mortality rate is about 20%[26].

1.5. LABORATORY DIAGNOSIS

The diverse clinical presentations of this disease make it essential for diagnosis by direct microbiological observation of samples demonstrating the presence of leptospires, further culturing in EMJH semisolid medium and serology by ELISA and MAT[27]. Further, the confirmation of serovars is done by cross adsorption agglutination test (CAAT) based on the lipopolysaccharide antigen and this technique is likely to be a confirmatory for culture so far[28]. In Dark field microscopy, the typical motility of the leptospires in the clinical sample (blood, CSF, urine or peritoneal fluid) observed with dark background, may aid in early diagnosis. Artifacts like lysed RBCs, fibrils may however, be mistaken for leptospires. So it is not recommended that dark field microscopy is the only diagnostic procedure to confirm the cases[29]. As a presumptive diagnosis, inclusion of IgM ELISA, latex agglutination test, indirect haemagglutination assay, lateral flow and dipstick is recommended[30 31 32 33].

The confirmation of the samples and cases are done by culturing in EMJH semisolid medium by inoculating blood or other clinical materials. A fourfold or greater rise in titre or seroconversion in microscopic agglutination test (MAT) on paired samples obtained atleast 2 weeks apart is very much useful to understand the stages of the illness and also apply as epidemiological tool to determine the cases in the living modalities[34 32].

1.6. TREATMENT

Leptospirosis is a curable disease; early diagnosis and prompt treatment help the patients to recover earlier. An effective course of treating the patients is still conspicuous unsolved problem[35 24]. In the initial treatment, doses of benzylpenicillin may be helpful but Jarisch-Herxheimer reactions may occur after the start of penicillin therapy[36]. In renal management, treated orally with antibiotics such as doxycycline are effective. Alternatively, in doxycycline contraindicated patients, tetracycline, ampicillin or amoxicillin may helpful. If the patients showed allergic reactions to penicillin, usage of third-generation cephalosporins including Ceftriaxone, cefotaxime and quinolone antibiotics may also be effective. Supportive management of patients including monitoring and additional care like dialysis, mechanical ventilation etc is adequate.

Prophylactic measures are helpful to reduce the infection rate thereby personal protective equipments are recommended. Expose to and handling the animals should be more cautious. The doxycycline prophylaxis may be useful in the case of exposures that are prone to get the infection[37]. In houses, rice mills and other places where more rodents are found must be aware about the rodent control and its management. The cleaning of rodent and other animal excreta should be standardized and in suspicion, immediate medical attention should be made[22]. The public have to aware about this infection and clinicians are also requested to suspect leptospirosis while pyrexia of unknown origin cases consulted[38 39]. The animals in the residential areas and domestic purposes should be vaccinated with serovar specific vaccines to avoid the spread of infections to humans.

2.0. AIM AND OBJECTIVES

AIM

The present study determines the seroprevalence of *leptospira* specific antibodies in several risk groups including canine pet owners, farmers, butchers and laboratory workers. Testing the pet dogs for the presence of Leptospires and its specific antibodies by culture and serology.To correlate the results with their owners to know any possibility of transmission from their dogs to them.

OBJECTIVES

- To evaluate the seroprevalence of Leptospires in canine pet owners and other human risk groups like farmers, butchers, laboratory workers in the study
- To correlate the epidemiological data (age, sex, occupation, duration of contact with pets) of the study subjects with the seroprevalence
- To confirm the laboratory identified leptospiral cultures by Cross Absorption Agglutination Test (CAAT)
- To perform standard serological techniques for determining Leptospirosis prevalence
    a. Genus specific Enzyme Linked Immuno Sorbant Assay (ELISA)
    b. Serovar specific Microscopic Agglutination Test (MAT)
- To compare and estimate the sensitivity and specificity of the serological techniques and to compare the serovar specificity for culture and MAT among pet dogs
- To analyse and compare the specificity of the infectious moiety among pet dogs and its owners

3.0. REVIEW OF LITERATURE

3.1.1. HISTORY

Leptospira was isolated and identified as the causative agent of the severe human syndrome Weil's disease about 100 years ago almost simultaneously, but independently, by workers in Japan and Europe[40]. Since that time leptospires have been isolated from almost all mammalian species globally, with leptospirosis now recognized as the most widespread zoonosis and also a major cause of disease in many domestic animal species. The history of leptospires was first reviewed and confirmed[10]. The contagious nature and microbial origin of this infection was proved in nineteenth century thereby this spiral shaped bacteria was named as *Spirochetae icterrohemorragiae* [41]. The name *Leptospira* was proposed later by understanding the morphology and movement and texted in Bergey's Manual of Systematic bacteriology[42]. After that, most of the research in leptospirosis in the next two decades related to the discovery of new serovars.

In USA, outbreak of leptospirosis in cattle and pigs due to *L. pomona* induced research related to nutrition and cultivation of leptospires[43]. Researchers discovered that the only carbon and energy sources were the long chain fatty acids that should be given in a detoxified form by adding serum or serum albumin[44].

Nowadays many sera enriched and chemically defined media are available for cultivating leptospires. Historically important development in the last 15 years dealt with Lipopolysaccharide antigen involved in immunity, flagellar gene code and other

important proteins in leptospiral genome. Genetic speciation can be done by molecular techniques. The current system of genetic classification was adopted in 1994[45].

3.1.2. EPIDEMIOLOGY

Leptospirosis is endemic in many parts of the world and causes frequent outbreaks in favorable situations. Leptospiral infections tend to occur as individual or small cluster of cases or large outbreaks or epidemics.

3.1.2.1. Predisposing factors:

Urban and rural areas both can be affected by Leptospirosis. In developing countries, a contaminated environment due to factors such as overcrowded slums, inadequate drainage and sanitation facilities for man and animals, presence of stray dogs, cattle, pigs, domestic rats, bandicoots, poor condition of slaughter houses and people walking bare foot contribute to the spread of the illness[11].

3.1.2.2. High risk groups:

Persons of all ages and races are susceptible. Adult men who tend to work in high-risk jobs are more frequently infected. The high-risk groups are workers in rice fields, cane fields and other agricultural crops and animal husbandry staff. In addition, workers in sewers mines, military personnel are also at risk[46].

3.1.2.3. Reservoir of Infection:

Rodents, domestic & wild animals are the reservoir of infection. Most of the infected wild animals and domestic animals that spread leptospirosis do not appear ill. The carrier animal shed leptospires intermittently. They shed leptospires for months and sometimes for life[47] (WHO, 2003). Domestic animals such as cattle, dogs, and pigs

may act as carriers for several months (temporary carrier) while rodents usually remain carrier throughout their life (permanent carrier) [48]. Rodents are therefore considered as the major reservoir of infection (Figure 1). These organisms can survive for 6 hours in dry soil and for 6 months in flooded condition.

Figure 1: Major reservoir of infection – Rodent

3.3. Mode of transmission:

The illness commonly occurs during the monsoon months. They enter the host through the abrasions of the skin of the feet or intact mucous membranes of eye, throat and gut[11]. The reservoir animals vary from area to area. In some areas it is raccoons, in others, skunks, in some, rats. Leptospires are excreted in the urine of the animals. The infection is transmitted when they wade through stagnant rainwater contaminated by infected urine of animals[47]. Therefore the more important epidemiological factors are rainfall and contact with contaminated environment[11].

A resurgence of leptospirosis in dogs reported in some areas of North America was thought to be due to exposure of pets to increased populations of urban wildlife, with a shift in prevalence of serovars from canicola and icterohaemorrhagiae to

grippotyphosa[49]. The primary reservoirs of the latter serovar is raccoons, opossums and skunks, with dogs, cats, humans and other animals being incidental hosts. Under diagnosis and under reporting of the disease were frequent, due to asymptomatic infection and the wide range of symptoms[50]. The following figures describe the risk of exposure of dogs to leptospirosis and further cycle to human infections (Figure 2, 3 and 4)

Figure 2: A dog – maintenance host

Figure 3: Mode of transmission

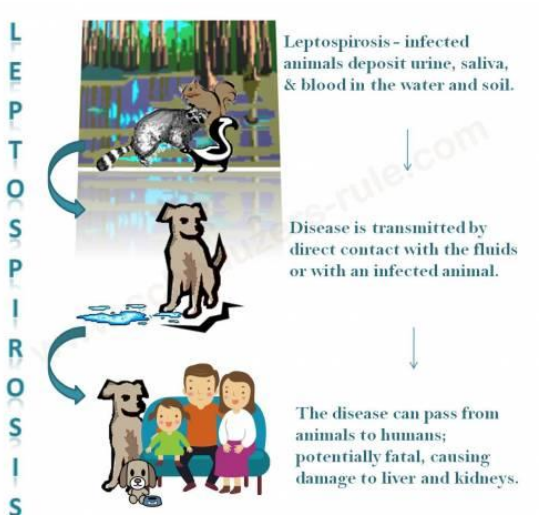
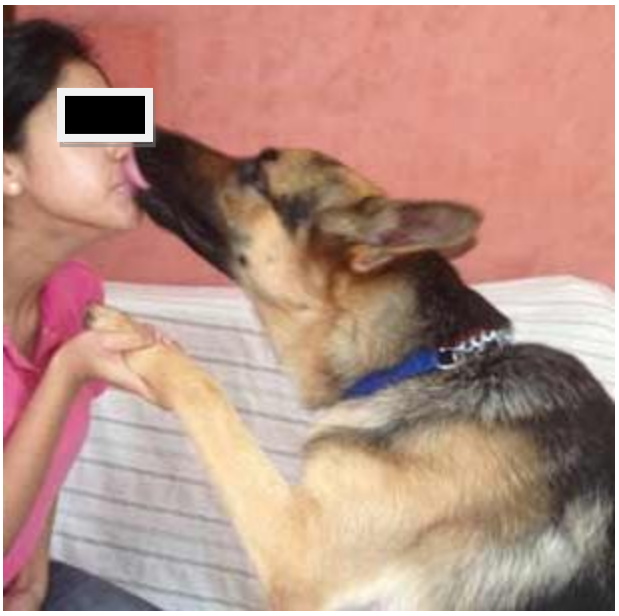


Figure 4: High risk of leptospirosis due to close contact with dogs



The number of cases in a region may vary year to year due to various factors such as rainfall, flooding and animal infections[46]. A fever outbreak was recorded recently at a village in Sivagangai district, Tamil Nadu in September 2014 and was investigated to identify the etiology and epidemiology. Eleven affected individuals who had both leptospirosis and Dengue were interviewed for risk factors. They gave history of either wet land agriculture practice, walking bare foot during a monsoon, association with pet animals or few associations with drinking water contamination[51].

3.4. OUTBREAKs

In the past 15 years, a numbers of outbreaks have occurred in many parts of the world. In 2000, in Malaysia 80 fatal cases were reported associated with international sports meet[52]. In the year 2002, 143 cases reported in Orissa, India out of which 11 were fatal[53]. In 2003, out of 204, 27 were fatal in India[54]. In Kenya 141 cases were reported out of which 12 were fatal in high school children[55]. In 2004 and 2005, 80 & 65 cases were reported respectively from Russian federation which was followed by swimming in a

river. Further, a Washington State report cited a significant number of canine cases of leptospirosis between October 1, 2004 and June 15, 2006.In 2006 & 2007 India had major outbreak with 258 & 1,516 cases respectively. In the year 2007, Argentina had 400 cases; Dominican Republic had 200 cases out of which 25 were fatal. During the year 2008, major outbreak occurred in Srilanka with 4,500 cases in which 1,150 cases were fatal[56].

In India, outbreaks of 'Andaman hemorrhagic fever' were first reported with hemorrhagic manifestations in 1988, and identified as leptospirosis in 1993 where 66.7% of the victims had significant titers of antibodies against *Leptospira* [57]. Most outbreaks of leptospirosis were reported in coastal regions like Gujarat, Mumbai, Kerala, Chennai and Andaman islands[58].

High rates have been reported from Valsad district (Gujarat) for several years and occur mainly during October to November. The main reservoirs in this country are rats, pigs, cattle, bandicoots, dogs and cats[18] (Patel *et al.*, 2006). In India, urban leptospirosis has been reported from Chennai and Mumbai while rural leptospirosis has been reported from Gujarat, Kerala and Andamans[59].

In 1994, an increase in the number of individuals with uveitis was noted at Aravind Eye hospital, Madurai, India after an epidemic of leptospirosis in South India. The epidemic followed severe flooding of the Tamil Nadu state in the autumn of 1993. Thirty seven out of 46 patients (80%) had leptospiral DNA and 33 out of 46 patients (72%) had positive serology[60]. Thirty eight acute renal failure cases with clinical

suspicion of leptospirosis were screened from July to November 1996 and 27 (71%) seropositive cases were diagnosed by MAT[61].

3.5. PREVALENCE

The Caribbean and Latin America, the Indian subcontinent, Southeast Asia, Oceania, and to a lesser extent Eastern Europe, are the most significant foci of the disease, including areas that are popular travel destinations[62]. The disease is absent from Canada and has been removed from the notifiable diseases list in the USA, where few annual cases occur, randomly related to recreational activities in fresh water except Hawaii with an estimated annual incidence of 12.9 per million population[63]. In Europe, the overall situation has not changed significantly over the past decade. Data from the Portuguese Ministry of Health for the period 2001—2005 showed an increasing incidence in the Azores islands, which account now for more than 50% of the annual cases. Underreporting is a major problem in evaluating the actual incidence of leptospirosis in many Asian countries. The Andaman and Nicobar Islands top the list of the most endemic areas of the world. The Seychelles islands possess the highest incidence worldwide, with annually reported cases and incidence remaining more or less constant from 1988 onwards according to Ministry of Health and Social Services reports and the annual incidence was above 1000 per million population in the 1995—1996 period[64]. In continuity with the hyperendemic Southeast Asia zone, Oceania also exhibits a significant burden of leptospirosis. In Australia, according to data from the Department of Health and Ageing, most cases are derived from Queensland, where the annual incidence is 28 cases per million populations, the remaining cases emerging from New

South Wales[65]. North Queensland is one of the world areas with the highest endemicity. In Africa 15% seroprevalence was demonstrated in a study in Gabon[66]. Prevalence surveys showed leptospirosis in 22.57% of FUO in Guwahati, Assam[67], 3.2% of febrile patients and 7.0% of febrile patients with jaundice in Delhi in1966[68]. Twenty three percent of patients hospitalized for febrile jaundice in Kolkata were positive for leptospirosis[69]. Further, seroprevalence was 8.8% in Chandigarh[70], 21.74% in Varanasi, 16.6% of sewer workers in Pune[71], 19.1% of tribal peoples on the Nicobar and Andaman Island[57], 23.6% of schoolchildren in the Nicobar and Andaman Islands[72], 32.73% of patients with FUO, 35.71% of farm workers and 39.47% of hepatitis patients were also affected and no prevalence observed in control groups[73].

Another study done by Tamilnadu Veterinary and Animal Sciences University between 1997 to 2006, showed seropositivity by MAT in human samples as 57.55%; highest (78.70%) during 1998-1999 and lowest (32.82%) in 2002-2003. Number of specimens received increased from 2054 in 1997-1998 to 10,014 in 2005-2006. This reflected on the increased awareness about the disease[23].

Most of the studies highlighted the gender and age wise seroprevalence. Samples received from October to December 2004 showed that the seroprevalence was more in males (57.30%) than in females (42.70%). In the study, five age groups were made separately for males and females including 0-5 years, 5-10 years, 10-20 years, 20-40 years and above 40 years. The seropositivity distribution for these groups among males was 11.41%, 12.11%, 18.16%, 40.16% and 18.16% and among female patients it

was 11.25%, 11.56%, 17.19%, 34.84% and 25.16% respectively. The mean seropositivity observed in this study was in patients above 20 years of age in both sexes[23].

A 10 year retrospective seroepidemiological study in North India showed a total seropositivity of 26.90%.Out of which, 30% seropositivity during 2000 to 2003 which decreased to less than 10% in 2009-2010. In a study from Nagpur[73], the prevalence of leptospirosis in patients being investigated for fever of unknown origin (FUO) was 32.73%. In another study from Chennai (Tamil Nadu), the year-wise prevalence of leptospirosis in 2004, 2005 and 2006 were 14.7, 24.9 and 32.3%, respectively[32].

The seroprevalence of leptospirosis in a study done in West Bengal has been found to be 14.45%. Male preponderance (63.6%) has been found in the study and 58.4% of the cases found in adults >15 years of age. The higher prevalence in males can be attributed to more frequent outdoor activities. The patients with 62 (80.5%) reactive cases were noted during the months of July to November (monsoon and post-monsoon season) in the study[74]. Another study which looked for the serological correlation of clinically suspected letospirosis patients in West Bengal showed seropositivity of 35% in suspected febrile patients[75].

To know the prevalence of leptospirosis among fever cases in private clinics, the Zonal Entomological team and Directors of Public Health and Preventive Medicine Chennai, performed a study in Villupuram District, Tamil Nadu, India. Blood samples were collected from three urban towns namely Kallakurichi, Villupuram and Thindivanam, from fifteen clinics, based on case definition of leptospirosis delineated by the National Vector Borne Disease Control Programme, Government of India. Samples

were tested with Macroscopic Slide Agglutination Test (MSAT) and IgM ELISA. There were 65 positive cases detected from 1502 blood serum samples in both MSAT and IgM ELISA. So, leptospirosis contributed to 4% of the fever cases from private clinics[76].

3.6. HIGH RISK GROUPS

Studies were carried out to analyze the prevalence of leptospirosis in high risk occupational groups. In 1993, a serosurvey of conservancy workers in Madras (using MAT) revealed a prevalence rate of 32.9%[77]. In 2004, a study was conducted for assessing the seroprevalence of leptospirosis among the high risk groups of Andaman Islands. Out of 611 sera samples from different high risk populations, 322 were positive by microscopic agglutination test (MAT) with an overall seroprevalence of 52.7%. The seroprevalence was highest among agricultural workers (62.5 %) followed by sewage workers (39.4%), animal handlers (37.5%), butchers (30%) and forest workers (27.3%). Among the control group the sero prevalence was 14.7%. Grippotyphosa followed by Australis were the common serogroups identified[78].

A study in North India between 2004 and 2008 showed Leptospirosis cases in rise from 11.7% to 20.5%. They observed cases occurance more commonly between the months July to October in each year of their study. Young adults were more commonly affected with the mean age of 32.6 years. Male preponderance was observed in this study. Major epidemiological risk factors noted in this study was wet environmental living conditions, lack of protective footwear, infestation of dwelling with rats, working in farm lands, contact with animals, especially cattle, bathing in public places, history of unprotected contact with dirty stagnant water, alcohol addiction and smoking[79]. In a

seroprevalence study conducted for risk group assessment, out of 30 veterinary doctors 2 (6.66%) were seropositive and 12(16.43%) out of 73 para technical supportive staff were seropositive. The seroprevalence in conservancy workers in Coimbatore was 12.58% in which the predominant serovar was australis[23].

A seroprevalence study was done for syphilis and leptospirosis among irula tribal communities of Tamilnadu to assess the risk factors associated with the disease in that particular population. Seroprevalence of Syphilis was 6.06% and seroprevalence of Leptospirosis was 56.97%. Irulas were generally designated as "Rat catchers" and may be the reason for higher prevalence in this community. The tribal environment, favorable for the survival of leptospirosis and more over the tribes association with domestic and wild animals might serve as a source for acquiring leptospiral infection[80].

3.6.1. Animals to Human Transmission

Worldwide, about 20% of cases of leptospirosis were thought to be associated with pets or rodents in and around the house. So, other than high risk occupation, rearing domestic animals like cattle, pigs and especially pets also carries the risk of spread of infection to human. This association is rarely reported in developing countries[4] . Reports of leptospirosis transmitted from pets to humans are available but it is not clear whether transmission was associated with illness in the animal. A case series of children with leptospirosis implicated exposure to dogs, but the dogs were not noted to be ill[81]. A case control study showed that dog ownership and the presence of rodents were the risk factors for leptospirosis in the context of flooding in a developing country[82]. There were cases of serious illness in children in the United States before serovar canicola was

controlled in dogs through vaccination. Vaccinated animals may still shed infectious organisms in the urine[83]. Dogs are considered as maintenance hosts for serovar Canicola. They are incidental hosts for other serovars and are a potential source of infection for human beings in contact with them. In some cases, the dog's urine is also a major source for the leptospiral infections[24]. They act as a link between the reservoirs of infection in the environment and the human beings. Not all dogs that are exposed to leptospirosis become visibly ill. Most of the pet animals are asymptomatic and shed leptospires in urine frequently[29].

The most common signs of the diseased dogs are fever and depression. These pets are cold, shivery and stiff. They may carry their tummies tucked up due to pain. Some drool and vomit and most of the dogs lose their appetite. Fever causes many dogs to drink excessively and this is not found in any other dog infections. Because symptoms vary so much between pets and because most veterinarians only see a few cases from time to time, it is common to miss the diagnosis on the first examination. .In carrier animals, leptospires will remain in the proximal renal tubules and sheded in urine every now and then. However, when these leptospires find their way into a new animal such as pet dogs, the harmonious relationship does not occur and may cause disease[4 13].

Exposure to various leptospiral serovars in veterinary staff and dog owners in contact with infected dogs was assessed in university of Bern Vetsuisse, Faculty of small animal clinic in the year 2007 and 2008. A total of 91 people (50 veterinarians, 19 technical staff, 9 administrative staff and 13 dog owners) who were exposed to dogs with leptospirosis were examined[84]. All the 91 human samples were seronegative for

leptospiral antibodies in MAT. The study also concluded the uncommon seroreactivity to leptospiral serovars in veterinary professionals and pet owners, even though they are exposed to dogs with acute leptospirosis[84].

3.7. Seroprevalence in animals

Indian report describing the predominance of various serovars of *Leptospira* in animals was noted. During 2003-2004, Hebdomadis (19.71%) was predominant followed by Australis (19.39%). During 2004-2005, Australis (21.67%) was predominant followed by Pyrogenes (13.95%) and during 2005-2006, Australis was highly prevalent (57.36%) followed by Pyrogenes (7.84%), Canicola (7.53%) and Hebdomadis (7.22%).

In the year 2006-2008, a prevalence study of leptospirosis conducted in dog, rodents and their possible role in transmission of human leptospirosis was analyzed in Mumbai. In the investigation conducted among 30 rodent populations, showed the predominance of Icterohaemorrhagiae followed by Australis.

Both the rodents and dogs had Pyrogenes as predominant serovar. Similar predominance of the same serovar was observed in a study on human samples during the same period[27].

This emphasises the rodent control measures and the prevention of contamination of food stuffs and water supplies with the excretions and secretions by the infected animals[27].

Seroprevalence of leptospirosis was investigated in animals using sera collected from six districts of Uttar Pradesh, India during a period of 2008-2010. Most of the

animals suffered from fever, jaundice, abortions, repeated bleeding etc. A total of 500 sera collected from cattle (250), buffalo (100) and dogs (150) were subjected to microscopic agglutination test, using two leptospiral serovars Viz. *L. icterohaemorrhagiae* and *L. grippotyphosa*. Seroprevalence was highest in dogs (9.3%) followed by cattle (8.4%) and buffaloes (6.0%). Animals of Badaun district showed highest seroprevalence for leptospirosis (10.9%). In all the animals seroprevalence was higher during rainy and autumn season in comparison to other seasons. Some studies suggested the need for continuous investigations and proper control measures for reducing high level of the prevalence of leptospirosis in animals[85].

In a study conducted in infected dogs, fifty two dogs with leptospirosis were seropositive in MAT and the serovars were Bratislavia (83%), Australis (83%), Grippotyphosa (35%), Pomona (23%), Autumnalis (23%), Icterohemorrhagiae (8%), Tarassovi (4%) and Canicola (2%)[84].

A study of 460 dog's samples which included various groups of dogs like owned, semi owned and stray dogs both vaccinated and non vaccinated confirmed that *Leptospira interogans* serovar icterohaemorrhagiae was the most common serovars and this population of dog had positive titre of 1:40. Out of them, 18.8% of positive cases of *L. icterohaemorrhagiae* were maintained in vaccinated dog population in this region. There is a perception among veterinarians that urban dogs have at lesser chances of exposure to leptospires than other dogs. In this study, they noticed that small breeds living in urban environment had higher titres to *L. interrogans* serovar icterohaemorrhagiae than other breeds. This was due to vaccination. However vaccine

induced titres rarely resulted in >300 and these titres only persist for 3–12 weeks after vaccination, falling below MAT titres of 1:100 [86](Senthil *et al.,* 2013). However, if the dogs were exposed to natural infection before vaccination, naturally the antibody titers would have been increased. This study supported the view that exposure to serovars grippotyphosa and autumnalis was common to household dogs and should be considered as a component of vaccines used in dogs. Where these serovars are known to be prevalent, inclusion of serovars pomona, grippotyphosa and autumnalis as part of canine leptospirosis vaccine should be considered for dogs[86].

Seroprevalence of leptospiral antibodies in canine population was studied in Namakkal district, Tamilnadu. In this study, various categories like vaccinated, unvaccinated, semiowned and stray dogs showed five leptospiral serovars known to be endemic including icterohaemorrhagiae, canicola, pomona, grippotyphosa, and autumnalis. Currently available leptospiral vaccines for dogs in India contain inactivated *Leptospira interrogans* serovars icterohaemorrhagiae and canicola. A serosurveillance study was conducted to provide further information on the changing epidemiological trend of canine leptospirosis infections in Tamilnadu. In that study, out of 42 vaccinated dogs, 24 (57%) were positive to one or more serovars. Of the 48 unvaccinated semiowned dogs, 10 (28.8%) showed positive agglutination to one or more serovars. Among the 34 stray dogs, 12 showed positive agglutination to one or more leptospiral antibodies. This study emphasized the changing trends in the epidemiology of leptospirosis with higher prevalence of serovar L. grippotyphosa in street dogs[86]. In a 2007 study, 24.9% of the examined unvaccinated healthy adult dogs, had antibody to

leptospirosis which indicated that they had been previously exposed to leptospirosis without their owners noticing the problem[87].

3.8. CULTURE:

The Spirochete *Leptospira* needs semisolid medium which comprise of carbon and nitrogen sources, certain growth factors and some inorganic salts. Rabbit serum was used in olden days for better growth of the organism. Bovine Serum Albumin (BSA) is a replacement forthat nowadays. The definitive diagnosis is obtained from culture and isolation of *Leptospira* using *E*llinghausen, *M*cCullough, *J*ohnson and *H*arris (EMJH) medium. Phospholipid is added to the medium to give good nutrition for leptospires[44]. These phospholipids were later replaced by Tween 80. The whole blood is inoculated (1 to 5 drops) into EMJH medium[44].

Leptospira survives in commercial blood culture bottles, although these samples require subculture to alternative media for bacterial isolation[83].

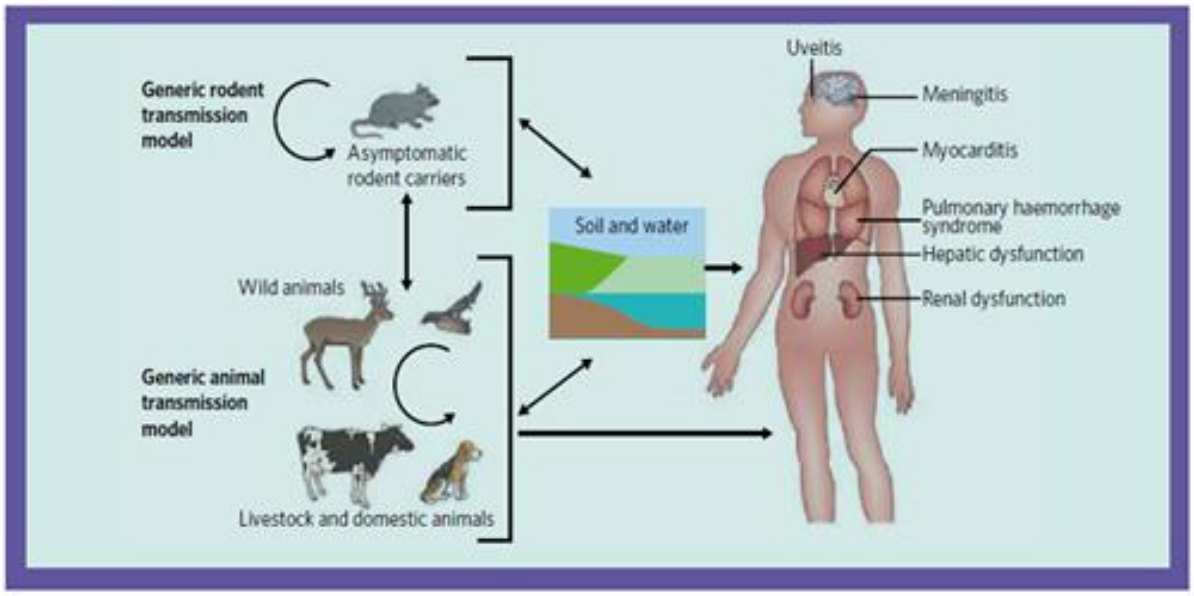
Culture is useful in outbreaks situation and in knowing global epidemiology. But it needs prolonged incubation period before discarding it as negative.

The microscopic agglutination test provides a broad idea of the serogroups in a given geographic area, but in one study, the predominant serogroups, at a titer of ≥100, correctly predicted less than 50% of serovars[88]. Given the need for isolation of *Leptospira* strains and the development of strain collections associated with human disease were well evaluated by studying the patients presenting to hospital with a febrile illness in a tropical region where leptospirosis is endemic, to evaluate which blood sampling strategy is associated with the highest yield of *Leptospira* .

3.9. CLINICAL FEATURES

Leptospirosis is an acute febrile illness with headache, prostration, myalgia (particularly calf muscle) and associated with any of the following symptoms and signs like conjunctival suffusion, reduced urine output or oliguria, jaundice, Cough, haemoptysis, breathlessness and haemorrhages in to the intestinal tract and lung parenchyma. Further it results in meningeal irritation, cardiac arrhythmia leading to failure and skin rash[4]. Leptospirosis initially causes fever, further in established disease it causes multiorgan dysfunction to multi organ failure (Figure5).

Figure 5: Multiorgan involvement in leptospirosis



The course of leptospirosis consists of two distinct clinical phases: septicemic and immune phase. Humans typically become ill after exposure to leptospires within 7 to 12 days.

Septicemic phase - The septicemic phase is the first phase of the disease which is otherwise called as leptospiremic phase because the bacteria may be isolated from blood cultures and cerebrospinal fluid (CSF).

It is characterized by a flulike illness with nonspecific symptoms with sudden onset of high fever, headache, myalgias (classically involving the paraspinal, calf and abdominal muscles) and conjunctival suffusion [92 93].

Immune phase - The second stage is called the immune phase -leptospiruric phase- circulating antibodies can be detected and the bacteria can be isolated from the urine [94 46]. Aseptic meningitis is one of the most important clinical syndromes that can occur in 80% of patients during the immune phase. Renal symptoms, such as uremia, azotemia, pyuria and hematuria, may occur[94 95]. Pulmonary manifestations, although usually benign, can be potentially life threatening and range from chest pain, cough and dyspnea to pulmonary hemorrhage or acute respiratory distress syndrome[96]. An increase in liver enzymes (up to five times normal) with a disproportionately high total bilirubin has been described as a prognostic indicator in leptospirosis[78]. Varying degrees of jaundice, pancreatitis, hepatomegaly and myocarditis can also occur[92 93].

Weil's Disease

Patients can present with high fever (>40°C), significant jaundice, renal failure, hepatic necrosis, pulmonary involvement, cardiovascular collapse, neurologic changes and hemorrhagic diathesis, with a variable clinical course. It can occur at any time during acute leptospirosis as a single, progressive illness[97 98].

Renal tubular necrosis is one of the most severe form of this leptospirosis in Weil's disease[94 95]. In previous days, acute pulmonary hemorrhage was the major cause of death in severe Leptospirosis[96].

Hepatic dysfunction is usually mild and reversible. Liver enzymes like SGPT, SGOT and Alkaline phosphatase will be elevated along with increased bilirubin levels, both indirect and direct bilirubin levels are elevated.

Variable degrees of thrombocytopenia have been reported with leptospirosis.

Weil's syndrome has a mortality rate of 5% to 10%. Important causes of death include renal failure, cardiopulmonary failure and widespread hemorrhage.

The clinical profile of Leptospirosis was analysed in a study in North Chennai South India. Out of 106 patients with positive Faine's criteria, males were 69, females were 37 & mean age was 31.2 years. Cases were reported throughout the year, 50.7% cases were noted during rainfall. The contaminated environment (95.2%) and animal contact (94%) were important epidemiological risk factors. Contaminated environment includes inefficient garbage disposal attracting rodents[100] and stray dogs, cattle, inadequate drainage facilities leading to stagnant water and wet contaminated environment, walking barefoot, absent indoor toilet and rearing domestic cattle & other animals[101].

Outdoor manual workers were the important occupational risk groups. Fever, headache, myalgia were the common clinical manifestation. Jaundice in 17.8% and renal failure in 10.3% were important complications. Anicteric leptospirosis was the

common clinical presentation 82.2%[101 102]. Previous studies from Chennai revealed that jaundice & renal failure were the important clinical features.

This is due to screening all patients admitted with fever for leptospirosis utilizing Macroscopic agglutination test(MSAT). This study, utilizing modified Faine's criteria has identified anicteric leptospirosis as the common presentation[102].

A case report in Hyderabad in the year 2013 explained the clinical and laboratory manifestations of two cases, a 16-year-old girl student and a 27-year-old male, a cattle farm owner. The predominant clinical finding was icterus with or without subconjunctival haemorrhage with other non-specific signs and symptoms. Exposure to potential sources of *Leptospira spp*. was contaminated water and direct contact or possible infection from contaminated environment[103].

Thrombocytopenia is an important contributory factor in the pathogenesis of bleeding diathesis in leptospirosis. It is important to anticipate and recognize this thrombocytopenia early in the course of disease so that appropriate steps can be taken to prevent it and to treat it with platelet transfusion when it developes[104].

3.9.1. ASSOCIATION OF CO INFECTIONS

As Leptosirosis can occur as differential diagnosis of acute febrile illness, other and co infections should be looked for before treatment. They are malaria, dengue, hepatitis A, hepatitis E, scrub typhus. The co-infections looked for in this study were malaria, dengue/ dengue hemorrhagic fever (DHF), hepatitis A and hepatitis E. In most of studies, the co infections of leptopsirosis were observed in a descending with hepatitis E,

malaria and dengue[105]. A study highlighted that one patient had co-infections of dengue fever and hepatitis E, while another patient had co-infections of malaria, dengue fever and hepatitis E[32 106 107].

A study was carried out to estimate the seroprevalence of Leptospirosis, Enteric Fever and Dengue in cases of acute febrile illness. Serum samples from 100 febrile patients were tested for *Leptospira* using Microscopic Agglutination Test (MAT), *Salmonella typhi* by Widal rapid and tube agglutination test, Dengue virus by IgM ELISA. This study also compared and analyzed the demographic characteristics. 21 patients were positive for leptospirosis (21%), 17 for Typhoid (17%) and 8 for Dengue (8%). Four patients had Co-infections. Two of them tested positive for leptospirosis and Typhoid, one for leptospirosis and Dengue. This was explained as because all the three infections were endemic in Chennai and the patients would have had infections with one or two causative agents causing acute febrile illness. 48% patients reported 1-5 days fever, 52% above 5 days, 86 % patients had intermittent fever. The positivity rate of leptospirosis among febrile patients was 21%[108].

Recently mixed fever outbreak was recorded at Kirungakottai village in Sivagangai district, Tamil Nadu, South India, in September 2014. The outbreak was investigated to identify the etiology and epidemiology of two febrile diseases Dengue and leptospirosis. The researchers also estimated the magnitude of leptospirosis outbreak and control measures were undertaken in the affected village. Out of 145 fever cases, 15 dengue cases and 7 leptospirosis cases were recorded from the village Kirungakottai. All

age groups were affected, but no death has occurred. 4 individuals were infected with both dengue and leptospirosis[51].

3.11. LABORATORY DIAGNOSIS:

As clinical presentations of this disease vary widely, laboratory confirmation is important for the diagnosis. Microbiological diagnosis aims at demonstration of the leptospires (direct evidence by wet mounting dark field microscopy), culturing in EMJH medium or by demonstrating antibody response to them (indirect evidence by ELISA and MAT).

Direct Evidences [109]

1. Demonstration of leptospires or their products:
    - ❖ Microscopy- Dark-field microscopy, Phase contrast microscopy
    - ❖ Staining-Silver staining, Immunofluorescence, Immunoperoxidase
    - ❖ DNA hybridization, Polymerase chain reaction
2. Isolation of leptospires
3. Animal Inoculation

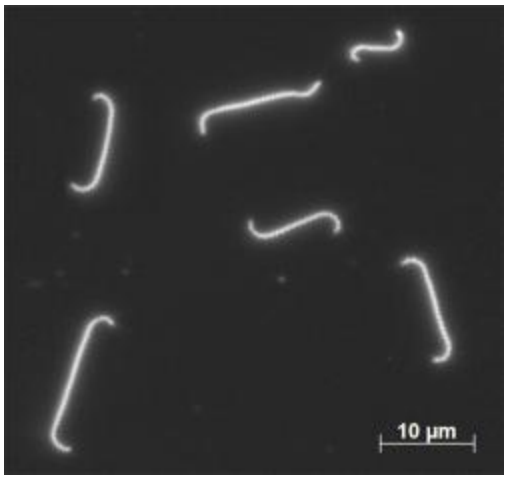
Indirect Evidence [110]

1. Detection of antibodies to leptospires
    a. **Genus specific tests -** Macroscopic agglutination test (MSAT), Indirect fluorescent antibody test (IFAT), Indirect haemagglutination test (IHA), Counter immuno electrophoresis (CIEP), Complement fixation test (CFT) Commercially available ELISA, Microcapsule agglutination test (MCAT), Lepto-Dipstick,

b. **Serogroup/ serovar specific tests -**Microscopic agglutination test (MAT), Serovar specific ELISA (rare in India, done only in Regional Medical Research Centre, Port Blair).

Dark ground microscopy from plasma is a simple economical method for early diagnosis. The typical motility (cockscrew or translational motility) of the leptospires (Figure 7) in the clinical sample (blood, CSF, urine or peritoneal fluid) observed with dark field microscopes aid in early diagnosis when correlated clinically[90]. Sensitivity of the test varies from lab to lab. MAT is a widely used reference test for leptospirosis but it has its limitations. It is inadequate for rapid case identification as it requires analysis of paired sera and can be performed in few reference laboratories. Moreover, the prevalent serovars in a particular geographic area must be known as it is cumbersome to test for all 200 serovars of *L.* interrogans. Double centrifugation of the sample at low speed to separate the cellular elements and high speed to concentrate the leptospires are preferred. Artifacts like lysed RBCs, fibrils, etc may however, be mistaken for leptospires. So, Dark field microscopy is not recommended as the only diagnostic procedure for leptospirosis confirmation[47].

Figure 6: Dark ground image of leptospires



Magnification : high power 40X

Leptospira can be cultivated in many synthetic and semi synthetic media[111 112]. Media used for cultivating leptospires are as follows:

Liquid

- Serum enriched- Korthof's, Stuart's Vervoort's
- Serum replaced by albumin and Tween - EMJH, PLM-5, *Leptospira* 5x, Chemically defined medium - Shenberg's, Vogel and Hunter.

Semisolid

- Serum enriched- Fletcher's, Noguchi's
- Serum replaced by albumin and Tween- Semisolid EMJH

Recently most of the clinical medicine depends on serology for confirming the clinical diagnosis of leptospirosis. Immune response of specific IgM, IgG, and IgA class antibodies in human leptospirosis in acute phase and in convalescence was analyzed by Genus specific ELISA test. A study reported that two groups of patients, 57 in the acute phase and 10 during convalescence were followed up. The mean follow up was 10.5

months. IgM class antibodies were detected from the day 2 of symptoms and persisted in 100% of patients till 5th month, in 66.7% of patients up to the 7th month and in 50% up to the 12th month from the onset of symptoms. But IgG class were first detected on the 7th day of symptoms in 9.1% of patients which peak (87.5%) between the 2nd and 3rd month, and it was not detected at all in one patient. IgA class antibodies were detected on the 5th day of symptoms in 7.7% of patients, and up to 9th month in all patients. In 12th month, they were detected in 83.3% of patients. They emphasis that an anti IgA ELISA could be of better value in human leptospirosis seroprevalence studies[113].

The Microscopic Agglutination Test (MAT) is the serological test used in reference laboratories. Antibody detected by MAT will appear on 6th or 7th day after development of symptoms and peak by 4th week, but detectable titers will be present for years[114 90].

Interpretation of the results is done with paired specimens collected at the appropriate times. So results are not available quickly for the patient management.

Till the serology developed, MAT was considered as the gold standard method to diagnose leptospirosis. Patients' epidemiological data about Leptospirosis can be obtained by this test. Commercially available serological tests include an immunoglobulin M enzyme-linked immunosorbent assay (IgM ELISA), an IgM dipstick assay (LDS), an IgM dot-ELISA dipstick test (DST) and the indirect hemagglutination assay (IHA) are also helpful with certain limitations and applications but MAT is highly sensitive and selective to evaluate the infection.

To prove the above statement, a study conducted in 2003 was analyzed with four rapid serologic tests for the diagnosis of leptospirosis, and the performance of each was compared with the current gold standard test - MAT.

DST and ELISA were based on EIA technology, LDS utilized colloidal dye. IHA was a biologic assay with hemagglutination as the endpoint. Microtiter plate ELISA gave a numerical endpoint as optical density value, all other assays required interpretation of color intensity or agglutination and thus a degree of subjectivity. But in DST, positive result was defined by the appearance of two or more discrete dots against a white background with less subjective bias. These variations in sensitivity, precision, stability, accuracy, limitations and specificity confirmed that MAT is the gold standard test.

The sensitivities for detection of leptospirosis cases were 93.2% by LDS, 92.5% by DST, 86.5% by ELISA, and 79.0% by IHA.

A highest concordance was observed between MAT and DST. False positive results were frequent (>20%) in sera from individuals with Epstein-Barr virus, Human Immunodeficiency Virus and periodontal disease and from healthy volunteers tested by LDS[115].

They observed that the second-generation assays like DST and ELISA are highly sensitive for early acute-phase sera than the reference or first-generation methods, MAT and IHA. It has also retained the high specificity and this will improve the rapid detection of leptospirosis in the field level[115].

Two cases of acute febrile illness with suspicion of leptospirosis were reported in Hyderabed. The diagnosis was confirmed by positive leptospiral specific IgM antibodies (Leptocheck), that was confirmed by gold standard MAT (microscopic agglutination test) as serogroup Tarassovi 1:1600 in student and Autumnalis 1:3200 in cattle farm owner using a panel of 19 live leptospiral serovars and positive anti- Hemin binding protein A(anti-HbpA) IgG antibodies. Culture was negative for blood and urine in both cases. Clinical improvement observed in 5-7 days after starting 10-day course of oral doxycycline 100 mg/twice/day. They concluded that rapid dipstick assay Leptocheck, had high sensitivity (86.8%) and specificity (92.7%) [116] and has role in early diagnosis of Leptospirosis. As HbpA-IgG ELISA indigenous kit had shown considerable potential in the identification of positive cases of leptospirosis, they emphasized the use of HbpA-IgG ELISA as a screening test[117]. Some of the studies highlighted that the first and second generation assays were having high sensitivity than MAT, but WHO clearly depicted that MAT should be confirmatory and also suggested to finalize the other serological evidences by comparing with MAT report[47].

In a study conducted in Madurai, a total of 60 serum samples from known leptospiral uveitis patients were analyzed by HbpA IgG ELISA. Results were compared with gold standard Microscopic Agglutination Test (MAT). HbpA is a hemin binding protein specific for *Leptospira*. This antigen was used in this study for the serodiagnosis of leptospiral uveitis. Compared with MAT, which gave the seropositivity of only 50%, anti HbpA IgG ELISA was detected in 92% patients[118].

Advanced molecular biological techniques like PCR were also studied in the diagnosis of leptospirosis. In 2000 to 2010, a study was conducted with more than 4000 patients by IgM ELISA. Out of total 391 samples, 192 IgM ELISA positive blood and urine samples were cultured for leptospires. MAT was performed for 8 serovars. PCR was performed in 115 blood and 38 urine samples. Of the 391 serologically positive patients it was observed that 226 cases (58%) were males and 165 were females (42%). The predominant serovars observed by MAT analysis were L. tarassovi (32 %) and L. australis. Among the IgM ELISA positive cases, PCR was performed on 115 blood and 38 urine samples. Ten samples each of blood (8.7%) and urine (26.31%) were positive by PCR. Positivity of PCR in urine was found to be higher than blood. In IgM ELISA negative cases, PCR was performed in selected blood and urine samples. PCR from blood was positive in 14 of 136 (10.29 %) cases, whereas PCR from urine was positive in 12 of 46 (26%) cases. Again, positivity of PCR in urine was found to be better than in blood[119].

In West Bengal, India Human leptospirosis was considered as an emerging problem. They analyzed 83 suspected fever cases with signs and symptoms of leptospirosis. Blood samples were tested for antibody against *Leptospira* by Immunochromatography and ELISA. IVD *Leptospira* IgM Microwell ELISA test kit (USA) was used to confirm the results. IgM ELISA, which uses *Leptospira* patoc 1 strain, is a standard serological test for early diagnosis of leptospirosis. IgM ELISA showed a positivity of 14.45% in this study. Antibodies did not reach detectable levels

until the 2nd week of illness[75]. An outbreak of leptospirosis in Mumbai in 2002 showed a positivity of IgM ELISA of 36.27%[120].

In a recent study in 2013, the accuracy of ELISA for the detection of human *Leptospira*-specific antibodies was evaluated. Meta analysis of 88 studies published in 35 articles was done. In convalescent stage of disease IgM ELISA had higher diagnostic accuracy. But they studied that regardless of the stage of disease IgM ELISA was the best choice. And also, negative ELISAs, whether IgG or IgM in the acute phase of disease do not rule out leptospirosis. It is due to the possibility of false negative results. In this condition a second blood sample or direct dark field microscopy or a direct method for leptospiral DNA can be done[121].

3.12. TREATMENT

The use of antimicrobial agents for treating leptospirosis has been restricted generally to penicillin and tetracyclines[122]. Other less frequently used antibiotics are beneficial but there is no proof of clinical studies. Erythromcyin and doxycycline may be helpful if there is penicillin allergy and to manage renal involvement. Studies proved that Doxycycline is useful as short term chemoprophylactic antibiotic. High dose of intravenous penicillin therapy may be useful for the patients in severe infections[123]. Current treatment of leptospirosis includes symptomatic support with or without direct antimicrobial administration, depending on the severity and duration of the symptoms[43]. The clinical application of antibiotics in the treatment of leptospirosis is depicted in table 1.

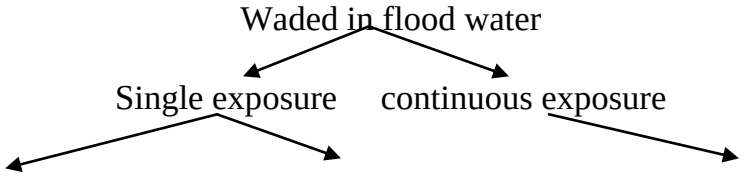
Table 1: Clinical application of antibiotics in the treatment of leptospirosis

Antibiotic	Route of administration	Daily dose	Duration
Penicillin G	Intravenously	4 – 6 million units/ 4 – 6 times	7 days
Piperacillin		4 – 8 gms/ 2- 4 times	7 days
Amoxycillin	Oral	1 – 2 gm/ 3 – 4 times	7 days
Cefpirome/Cefozopran/ Cefepime		2-4 gm/ 2-4 times	7 days
Streptomycin	Intramuscularly	1-2 gm/ 2 times	2-4 days
Doxycycline	Orally	200-400 mg/ 2 times	7 days
Minocycline	Orally/ Intravenously	100-200mg/ 2 times	7 days
Erythromycin	Orally	2-4 g/ 3-4 times	7 days

3.13. PREVENTION AND CONTROL MEASURES

Prevention of leptospirosis is not possible in the entire situation as it is ubiquitous in nature. Control measures that can be done are to limit the spread of leptospirosis in animals and from animals to humans. Chemoprophylaxis can be done with doxycycline, before and during exposure especially for high risk occupation groups and travelers to endemic areas.

Doxycycline chemoprophylaxis



No wounds,cuts or lesions With wounds cuts and lesion Withwithout cuts wound

Low risk	Moderate risk	High risk
Doxycycline 2 capsules 100mg / cap single dose within 24-72 hrs	Doxycycline 2 capsules 100mg / cap once a day for 3-5 days. Start within 24-72 hrs of exposure	Doxycycline 2 capsules 100mg / cap once a week until the end of exposure

In a seroprevalence study conducted in Villuppuram district of Tamilnadu. They offered the treatment of leptospirosis, active surveillance of cases in affected villages was done and attempted to rectify the spread through water distribution system by chlorination[124](Basker et al., 2014). In another study which included mixed fever outbreak of leptospirosis and dengue, apart from investigation to identify the etiology and epidemiology, the inclusion of other control and preventive measures was successful. Further, active fever surveillance, treatment of positive cases and entomological surveillance in the form anti larval and anti adult measures were done. Out of the cases included, 11 affected individuals were interviewed for the risk factors resulted with positive history of either wet land agriculture practice, walking bare foot during a monsoon, by association with pet animals or few associations with drinking water contamination[51].

Generally, vaccination against leptospirosis has been recommended for dogs, because of the prevalence of serovar icterohaemorrhagiae and canicola in rat population. Vaccination in dogs is considered as a long term protection against the establishment of

the renal carrier state, in order to protect other dogs, as well as humans. In this study, they described the ability of the new European tetravalent vaccine containing antigen from *Leptospira interrogans* serogroups Icterohaemorrhagiae, Canicola, Grippotyphosa and Austrlais to control the infection and renal excretion in digs at 12 months after vaccination. They concluded that a significant protective immunity was achieved in dogs 12 months afer a basic vaccination schedule of two doses against strains of serogroups Icterohaemorrhagiae, Canicola, Grippotyphosa and Austrlais[125]. Some studies also highlighted the utilization of human vaccines[126]. They are not FDA approved. Epidemiological studies should be carried out for proper evaluation of the endemicity of leptospirosis in every part of the country. Seroprevalence of leptospirosis in high risk population will give actual burden of the disease in that particular environment. So with this background it will be appropriate to study the antibody titre of the population who are vulnerable to get disease due to contiguity or closeness to leptospirosis affected pets and also due to high risk occupation.

Thus this study was designed to understand the seroprevalence of leptospirosis among pet dogs, its owners and other risk groups in Tiruchirapalli, Tamilnadu, India.

MATERIALS AND METHODS

4.0. MATERIALS AND METHODS

4.1.1. Study area

This study included areas in and around Thiruchirapalli, Tamilnadu, INDIA, which consist of more than 35% of the population of agricultural background.

4.1.2. Study Population

The study was performed at a tertiary care teaching hospital and also included private veterinary clinic and risk groups including farmers, butchers and canine pet 4owners (Table 2).

Table 2: Subjects included in this study

S. No	Risk Groups	No. of Samples
1.	Pet Owners (Canine Pet Owners)	37
2.	Farmers	71
3.	Butchers	14
4.	Laboratory Workers	20
5.	Pet Dogs	53

The subjects included in this study are farmers, butchers, canine pet owners and lab workers. The suspected pet dogs brought to private veterinary clinic which were investigated for leptospirosis were also included in this study

4.1.3. Study Design and Period

A prospective cross-sectional observational study was undertaken in 142 subjects (95 males and 77 females) with age range from 20-70 years. The subjects were

interviewed for history of close contact with pet animals, duration of contact in years (for canine pet owners) and history of working in wet agricultural fields (for farmers), history of contact with carcasses and with animal excreta (for butchers) and history of working with Leptospiral cultures in laboratory (for laboratory workers).

Thirty subjects from healthy population with no risk factors for leptospirosis were taken as control. Fifty three dogs of age 2 months to 15 years were included in the study to screen for the presence of leptospires and its specific antibodies. The number of canines and the number of pet owners were not matched in this study due to unwillingness of 33 pet owners. Thus 70 canines (including control) and 37 canine pet owners were included in this study. Seventeen dogs which received regular vaccination and which were not allowed to mingle with other dogs were also included as control. The study period was from April 2014 to March 2015.

4.2. Ethical Clearance

This study got approval from Institutional Ethical Committee (IEC) of Chennai Medical College Hospital and Research Centre (SRM group), Thiruchirapalli, Tamil Nadu, INDIA to include human subjects (Figure 7) and for animals permission obtained from private veterinary clinic . Informed consent in a vernacular language was obtained from all study subjects and from pet owners for inclusion of their pets in the study.

4.3.1.1. Samples included

Blood samples for leptospiral culture and serological testing were obtained from all the human subjects (142 nos and 30 controls) and canines (53 dogs and 17 controls). Two male patients admitted in the tertiary care teaching hospital with fever which

progressed to multi organ dysfunction were included in the study. Blood samples were collected and subjected to serology

4.3.1.2. Sample Collection

Blood samples of approximately 5ml were collected for culture and serology. A field side inoculation was performed on the leptospiral selective media aseptically. The remaining blood was allowed to clot, centrifuged and serum was separated. The serum samples were stored at -20°C until use. The urine sample was collected in a sterile container and transported to laboratory for culturing, direct dark field microscopy (DFM) and serology.

4.3.2. Blood Culture

Before collecting blood the cubital area is surface sterilized with 70% ethyl alcohol. By using sterile syringe and needle 5ml of blood is drawn from each subject by venupuncture. Two to four drops of blood (approx. 0.5ml) was inoculated in the presterilized McCartney bottle containing EMJH semisolid medium.

4.3.2.1. Preparation of EMJH Medium

The composition of EMJH semisolid medium base was depicted in the table 3.

Table 3: Components of EMJH semisolid medium Base

Constituents	**Quantity**
Leptospira medium base stock	252mg
Agar Agar	100mg
Tween 80	50µl
Distilled water	70ml

4.3.2.2. EMJH medium Base

The base EMJH contains sodium phosphate dibasic, potassium phosphate monobasic, sodium chloride, ammonium chloride and thiamine. This complex composition was prepared as stock solution and stored at 4°C until use. In this study, prepared hydrated EMJH media base was used (Himedia Laboratory – M1009). The composition of which is depicted in table 4. The p H was adjusted to 7.6 to 7.7 at 25°C.

Table 4: Composition of EMJH medium base stock

Constituents	**Quantity**
Sodium phosphate dibasic (Na_2HPO_4)	16.6gm
Potassium phosphate monobasic (KH_2PO_4)	2.172gm
Sodium chloride (NaCl)	38.5gm
Ammonium chloride (NH_4Cl)	5gm
Thiamine	100mg
Distilled water	1000ml

4.3.2.3. Nitrogen source (Protein supplement)

Incorporation of nitrogen source enriches the medium for the structural extension of leptospires. One percent BSA is added to the EMJH base medium. The 1 % BSA solution was prepared and sterilized by membrane filtration with the pore size of 0.45µ. This protein filtrate was added to the autoclaved EMJH media base for dispensing in sterile screw cap tubes.

4.3.2.4. Vitamin solution

The addition of vitamins gives better nutrition for the Spirochetes. Table 5 highlights the composition and preparation of vitamin solution.

Table 5: Preparation of EMJH medium Vitamin solution

Constituents	Quantity
Thiamine Hydrochloride	5mg
Nicotinic Acid	1mg
Cyanogobalamin	1mg
Distilled water	10ml

This vitamin solution is also sterilized by membrane filtration, to avoid degradation of vitamins by other modes of sterilization like autoclaving.

4.3.2.5. Mineral supplement mixture

The addition of minerals including calcium chloride, copper sulphate etc provide extensive and rapid growth of leptospires. This mineral supplement also maintains the pH of EMJH medium at constant level. The composition and preparation procedure of mineral supplement mixture is depicted in Table 6.

Table 6: Preparation of mineral supplement mixture

Constituents	Quantity
Calcium Chloride ($CaCl_2$)	1%
Zinc Sulphate ($ZnSO_4$)	0.4%
Copper Sulphate ($CuSO_4$)	0.03%
Magnisium Chloride ($MgCl_2$)	1%
Sodium pyruvate	20%
Mix 1.5ml 0f $CaCl_2$, 1ml of $ZnSO_4$, 100µL of $CuSO_4$, 1.5ml of $MgCl_2$ and 200µl of Sodium pyruvate	

4.3.3. Medium dispension

After preparing all the constituents, aseptically all are mixed together as depicted in Table 7.

Table 7: Preparation of EMJH semisolid medium

Constituents	Quantity
EMJH medium base	70ml
Protein supplement	30ml
Vitamin solution	5ml
Mineral supplement mixture	4.3ml
Selective agent (5FU) (For urine culture only)	1ml

Meanwhile, McCartney bottles are cotton plugged and autoclaved. The rubber lining present inside the lid was removed and a hole was made in the center by keeping the lid in upright position. After putting the hole the tube lining was pasted inside the lid using feviqwik, and then sterilized separately. Aseptically the lid was screwed on the bottle after removing the cotton plug. The EMJH semisolid medium of 5-7ml was dispensed in the sterilized McCartney bottles. Sterility checking was done by placing the medium in room temperature for 24 to 48 hrs.

This media is highly complex to prepare and to maintain. Due to the presence of proteins and vitamins in the medium the chances of contamination by environmental microorganisms are high. So, the uninoculated medium stored at room temperature.

4.4.1. Incubation

The inoculated EMJH semisolid media were incubated in room temperature for 2 weeks to 6 months in the dark and examined.

4.4.2. Preliminary Confirmation

Dingers ring was observed in the EMJH semisolid medium due to the presence of leptospires. As it is microaerophilic, it appears 1 cm below the surface of the medium as white ring formation, due to the high concentration of leptospires. An uninoculated tube containing EMJH media is maintained as negative control. A known leptospiral culture inoculated medium was kept as positive control. Approximately 2-6 weeks were required for the visible growth of leptospires. The thick dinger ring appears on the 3rd week and it disappears after that.

4.4.2.1. Wet Mount analysis from culture:

In dark field microscopy, the presence of leptospires were observed and analyzed. The prompt and referred observation on dark field microscopy showed the typical leptospires with hooks at both ends and vigorous translational and rotational motility. Further the dark field method was developed using thick wet mount with cover slip. The reason for using cover slip may be to avoid cross contamination and infection to handlers, avoid drying and for preparing thin wet mount.

4.4.2.2. Procedure:

A clear microscopic slide was taken in which the isolates were made as thin wet mount with cover slip, and observed under dark field microscopy for the presence of leptospires. The medium were examined microscopically at weekly interval up to 6 months. The observed slides were discarded properly in a beaker containing diluted disinfectant (1% hypochlorite solution).

4.4.3. Cross Agglutination Absorption Test (CAAT) Analysis from Reference Center

The dark field microscopy and dinger ring positive tubes were aseptically maintained at room temperature until the serogroup confirmation. These tubes were subcultured on to fresh EMJH semisolid medium and also transferred to 2 ml capacity serum vial and sealed by applying paraffin wax inside the lid. Further these vials were sealed with parafilm and cellophane tape. These packed samples were sent to WHO collaborating center of Diagnosis, reference, Research and Training in leptospirosis, Regional Medical Research Center (ICMR), Port Blair, Andaman and Nicobar Islands, INDIA for serotyping at serovar level by Cross agglutination absorption test (CAAT).

Flow Chart: Preparation, Dispension, Inoculation and screening of EMJH semisolid medium

Preparation of EMJH medium [**Table 4]**

↓

Preparation of EMJH base stock [**Table 5]**

↓

Preparation of 1% BSA

↓

Preparation of vitamin solution [**Table 6]**

↓

Preparation of Mineral supplement mixture [**Table 7]**

↓

Preparation of selective agent (for urine culture)

↓

Medium Dispension [**Table 8]**

Sterility check (room temperature for 24-48 hrs)

↓

Inoculation with 0.5 ml-1ml of blood/ urine

↓

Incubate at room temperature in dark

↓

Screen every 3rd day from 2 weeks till 6 months in Dark field microscopy

↓

Positive cultures subcultured in fresh EMJH semisolid medium every 3rd month

4.5. Serology

Serum was separated from all blood samples and stored at - 20°C until use.

Three groups of sera were used

1. Serum samples separated from study subjects (n=127). A .petowners (n=37), B. farmers (n=68), C. butchers (n=14), D. laboratory workers (n=5).
2. Sera from healthy population (n=30)
3. Positive control and Negative control sera (lyophilized) were obtained from Regional medical research center, WHO collaborating center for diagnosis, reference, research and training in leptospirosis, PortBlair, Andaman and Nicobar islands, INDIA. (The lyophilized sera were reconstituted with 100µl of phosphate buffered saline – for MAT)

Two serological tests were included in the study

1. Genus specific Enzyme Linked Immuno Sorbant Assay (ELISA)
2. Serovar specific Microscopic Agglutination Test (MAT)

In this study *Leptospira* IgM ELISA commercial kit was used (Panbio, Standard diagnostics, Republic of Korea, Lot No. 261006). This test is performed only for human subjects.

4.5.1. Principle of ELISA

Leptospiral genus specific antigen is coated in the polystyrene micro titre wells of this kit. If serum contains specific antibodies to *Leptospira* antigen they get attached to the micro titre well surface. Residual serum was removed by washing then Horse radish peroxidase conjugated with anti human IgM is added. After washing the chromogen tetramethyl benzidine (TMB) is added. The substrate was hydrolysed by the enzyme and

blue colour is formed. After stopping the reaction with stop solution containing 1M phosphoric acid, the TMB changes to yellow. This colour development indicates the presence of *Leptospira* IgM antibodies in the test serum.

4.5.2. Procedure:

Preparatory phase

1. All reagents in this Panbio kit are equilibrated to room temperature before commencing assay.

2. Required number of micro titre wells were removed from the foil sachet.

3. Materials provided

i. Wash buffer preparation- one bottle, 60 ml of 20X concentration of phosphate buffered saline (pH 7.2-7.6) with Tween 20 and preservative (0.1% Proclin). Crystallization may occur in low temperature. To correct this, the solution is incubated at 37°C until clear. One part of wash buffer diluted with 19 part of distilled water diluted buffer can be stored for one week at 2- 25°C.

ii. Sample diluents – two 50 ml pink colored ready to use solutions provided with the kit.this contains preservatives and additives and stable at 2-8°C until expiry.

iii. HRP conjugate anti human IgM - 50ml yellow colored solution. Horse radish peroxidase conjugated goat anti human IgM with preservatives and protein stabilizer which is stable at 2-8°C until expiry is provided.

iv. TMB chromogen- A mixture of 3,3’,5,5’- tetramethylbenzidine and hydrogen peroxide in a citric acid citrate buffer with the pH of 3.5-3.8 is provided which is stable at 2-8°C.

v. Reactive control – 100µl of human sera containing 0.1% sodium azide and 0.005% gentamycin sulphate which is stable at 2-8°C until expiry is provided in the kit.

vi. Calibrator - 400µl of human sera containing 0.1% sodium azide and 0.005% gentamycin sulphate which is stable at 2-8°C until expiry is provided in the kit.

vii. Negative control - 200µl of human sera containing 0.1% sodium azide and 0.005% gentamycin sulphate which is stable at 2-8°C until expiry is provided in the kit.

viii. Stop solution – 15ml of 1M Phosphoric acid which is stable at 2-8°C until expiry is provided in the kit.

Procedure:

Plastic disposable test tubes were used to dilute positive control, negative control, calibrator and test serum. One ml of sample diluent was taken in each labeled tube. To that 10µl of negative control, positive control, calibrator and serum were added as 1: 100 dilution in separate tubes. From this diluted mixture 100µl was pipetted in to their respective wells. Negative control was added in the first well followed by that positive control added. Further calibrator was added from 3rd to 5th wells in triplicate. The diluted serum samples were added consecutively after that; the plates were covered with aluminium foil and incubated at 37°C ± 1°C for 30 minutes. After first incubation, microtitre plates were washed with wash buffer (6 times) in ELISA washer. One hundred microlitre of HRP conjugated anti human IgM added to each well, covered and incubated at 37°C± 1°C for 30 minutes. After second incubation, plates were washed 6 times with wash buffer in ELISA washer. Then 100 µl of TMB substrate was added to each well, covered and incubated for 10 minutes at room temperature. After incubation 100 µl of

stop solution is added to each well. The blue will change to yellow. With the help of ELISA Reader, the absorbances of the wells were read within 30 minutes. Reading the microtitre wells at 450nm without reference filter may result in higher absorbance value due to background. So a dual wavelength spectrophotometer with the reference filter range of 600- 650 nm is used.

4.5.3. Calculation:

Cut off Value is the multiplication of average absorbance of the 3 calibrators and calibrator factor. The Index Value can be calculated by dividing the sample absorbance by cut off value. This can be changed to Panbio units by multiplying by 10. To validate the test, the criteria are negative absorbance <0.200; cut off value ≥2x Negative absorbance; reactive cut off ratio 1.1- 6.0.

4.5.4. Interpretation of results:

As per CLSI guidelines, appropriate QC practices were followed before interpreting the results. The interpretation of the ELISA results was calculated and analyzed as described in table 8 and 9.

Table 8: Index value of the test results

Index value	Panbio units	Results
< 0.9	<9	Negative
0.9 – 1.1	9 – 11	Equivocal
>1.1	>11	Positive

Table 9: Interpretation of the ELISA observation

Results	Interpretation
Negative	No IgM antibody detected

Equivocal	Test should be repeated or repeated with alternate method
Positive	Presence of detectable IgM antibody

Flow chart : IgM *Leptospira* ELISA Test procedure

Preparatory procedure
10 µl of each negative control, positive control, calibrator and samples are diluted in 1000µl of sample diluent in separate test tubes
Wash Buffer preparation procedure
1 part of buffer concentrate diluted with 19 parts of distilled water

ELISA test kit reagents equilibrated to room temperature before commencing the test

↓

Required wells are removed from the foil sachet

Kit Procedure

100µl of diluted negative control, positive control, calibrator and samples pipetted to respective wells (1:100 dilution) Cover the plate and incubate at 37°C for 30 minutes

↓

Wash with wash buffer in ELISA washer

↓

Pipette 100µl of Anti human IgM HRP conjugate in all wells

↓

Cover plate and incubate at 37°C for 30 minutes

↓

Wash with wash buffer in ELISA washer

↓

Pipette 100µ of TMB substrate in all wells

↓

Cover plate and incubate at room temperature for 10 minutes

↓

100µl of stop solution added to each well

↓

Read the absorbance within 30 minutes with ELISA reader

Calculation	**Validity of the test**
1.Cut off value = average absorbance of the 3 calibrator X calibrator factor 2.Index value = sample absorbance / cut off value 3.Panbio units = Index value X 10	Negative absorbance : < 0.200 Cut off Value : ≥ 2 X Negative absorbance Reactive / Cut off ratio : 1.6 – 6

4.6. Microscopic Agglutination Test (MAT)

Serial dilution of serum kept in contact with an equal volume of a well grown suspension of leptospires at a certain temperature for a certain period of time and read microscopically by estimating 50% agglutination as the end point titre of the reaction mixture.

4.6.1. Preparation of antigen

EMJH liquid culture was prepared for culture maintenance. The procedure for preparation of EMJH broth is the same as EMJH semisolid medium preparation except excluding the agar-agar. Five to six ml of stock liquid culture medium was maintained in the sterile screw cap containers. Fresh subcultures were made by inoculating 0.5 ml of live culture in to the tubes containing EMJH liquid medium. At the same time, culture was tested for viable leptospires under dark ground microscopy. The inoculated culture was incubated at 30°C and checked for the presence of growth after 5- 7 days. A well grown culture with the minimum density of 1 -2 X 10^8 leptospires / ml was used as

antigen. The incubated culture was checked for viability and density. If any doubt arises the culture was further incubated. The density can be determined by direct counting, spectrophotometer or by McFarland's scales.

Quality Control

Cultures used for MAT was checked against homologous anti sera frequently.

4.6.2. Reference Culture Maintenance

A battery of 12 serovars covering the range of serovars expected or likely to be present in the particular geographical area was used. The recommended panel of serovars was received from Regional Medical Research Centre, WHO collaborating center for diagnosis, reference, research and training in leptospirosis, Indian Council of Medical Research, Port Blair, Andaman and Nicobar Islands, INDIA.

Table 10: Serovars used in this study for MAT serology

Serogroup	Serovar	Strain
Australis	Australis	Ballico
Autumnalis	Bangkinang	Bankinangi
Canicola	Canicola	H. uterecht IV
Grippotyphosa	Grippotyphosa	Moskva V
Hebdomadis	Hebdomadis	Hebdomadis
Icterohemorrhagiae	Icterohemorrhagiae	RGA
Javanica	poi	Poi
Pomona	Pomona	Pomona
Pyrogenes	Robinsoni	Robinson
Sejroe	Sejroe	M84
Sejroe	Hardjo	Hardjoprajtno
Semaranga	Patoc	Patoc I

4.6.3. Preparation of serum diluent – 1% Phosphate Buffered Saline (PBS)

The phosphate buffered saline was used as a diluent to follow the dilution of test antibodies. Table 11 describes the procedure of phosphate buffered saline (PBS) preparation.

Table 11: Preparation of 1% Phosphate Buffered Saline

Constituents	Quantity
Diluent	
Sodium Chloride (NaCl)	8.5gm
Disodium Hydrogen Phosphate (Na_2HPO_4)	0.85gm
Pottasium Dihydrogen Phosphate (KH_2PO_4)	0.25gm
Distilled water	100 ml
pH – 7.2	
Preparation of microtitre well washer	
1% PBS pH 7.2	100ml
Tween 20	0.5ml

4.6.4. Procedure:

The serum samples were diluted with 1% PBS at the pH of 7.2.the serial two fold dilution of the serum were prepared to provide the dilution of 1:10 to 1:1280 (further dilutions were carried out if more than 1:1280 titre showed positivity). Along with the diluted serum sample, equal volume of antigen is added (1:1 ratio). These microtitre plates are covered with aluminium foil and incubated in room temperature in dark for 2 hours. After incubation one drop from each well was taken in slide, cover slip placed (avoid air bubbles) and examined under low power and high power dark ground microscopy. Visible clumps against a dark background could be appreciated from the positive titre wells. Microscopic reading was performed using an agglutination end point

of 50%. The most common serovar associated with the highest titre in MAT was recorded for seroprevalence study.

After triplicate analysis the microtitre plates and slides were discarded in 0.5% sodium hypochlorite solution.

Flow chart: Procedure- Microscopic Agglutination Test

Components
Live leptospiral (12 serovars) in liquid cultures
1% PBS solution with pH 7.2

96 wells microtitre plate was labeled for serovars, dilutions, positive control and negative control

↓

180µl of 1% PBS sol. Pipette to first row wells (8 wells)

↓

In all other 88 wells 100µl of 1% PBS was added

↓

20µl of test serum was added in the first row of wells containing 180µl of 1% PBS and mixed well

↓

By pipetting 100µl from it serial dilution was done, discarding the final 100µl of diluted serum

↓

100µl of Serovar specific *Leptospira* culture was added to each column

↓

Microtitre plate covered with aluminium foil

↓

Incubate for 2 – 4 hrs at room temperature in dark

↓

Serum - antigen mixture was prepared as wet mount

↓

Examine under dark field microscopy for agglutination

↓

Microtitre plates and slides were discarded in disinfectant solution

4.7. Comparative analysis

Initially the results of ELISA and MAT were compared. Later they were compared with culture results. The identified serogroups from MAT and CAAT (culture) were compared and evaluated. By this test results, the sensitivity, stability, precision and correlation of each method was done.

RESULTS

5.0. RESULTS

During the period of March 2014 to April 2015, 172 human subjects who were at risk of acquiring leptospirosis were included in this study. A total of 70 healthy pet dogs were also included to analyze the presence of leptospires in EMJH culture and antibodies in serological screening. Among the human subjects prevalence of leptospirosis was observed by studying the age and sex distribution, occupational analysis, duration of contact with pet dogs and duration of exposure to risk environment.

In a 14 month period of study, only early and single sera were collected. The culture results of leptospirosis in EMJH semisolid medium and serology by Enzyme Linked Immuno Sorbent Assay (ELISA) and Microscopic Agglutination Test (MAT) were analyzed.

5.1. Age wise distribution

The human study subjects of 172 belonged to canine pet owners no.37; farmers no.71; butchers no.14; laboratory workers no.20 and controls no.30 were included in this study. The age distribution ranged from 16 yrs to 60 yrs. The break up details of each group is shown in table 12.

Table 12: Age and Sex wise distribution of Subjects (n= 172)

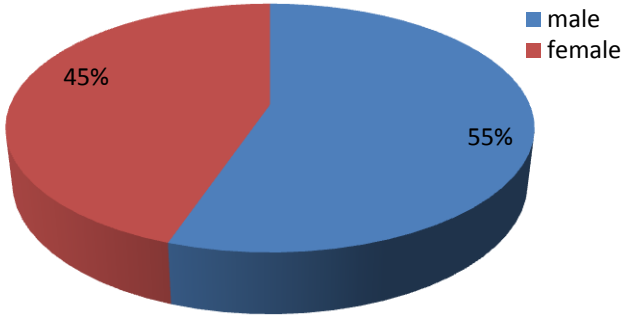
Study subjects	16-30 yrs		31-45 yrs		46-60 yrs		> 60 yrs	
	M^x	F^{xx}	M	F	M	F	M	F
Pet owners (n=37)	9	1	15	6	5	1	-	-
Farmers (n=71)	3	3	12	15	10	11	10	7
Butchers (n=14)	6	-	6	-	2	-	-	-
Lab workers (n=20)	-	2	9	8	-	-	1	-
Control (n=30)	4	17	3	6	-	-	-	-

[M^X - Male; F^{XX} - Female]

5.2. Sex wise distribution

Of the 172 subjects, 95 were males (55%), 77 were females (45%) and shown in the pie diagram (Figure 7) and the break up details are shown in the Table 12. In most of the cases, the numbers of females are less than males due to reduced risk.

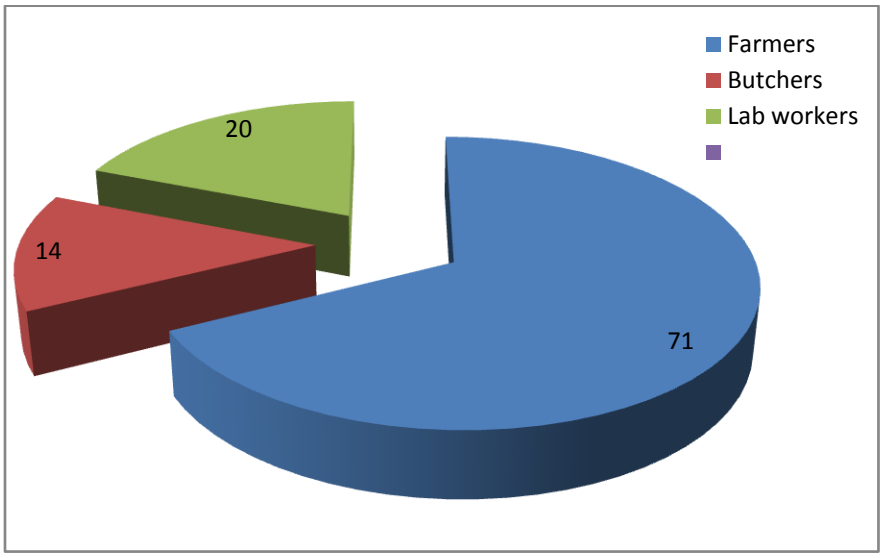
Figure 7: Sex wise distribution



5.3. Occupation wise distribution

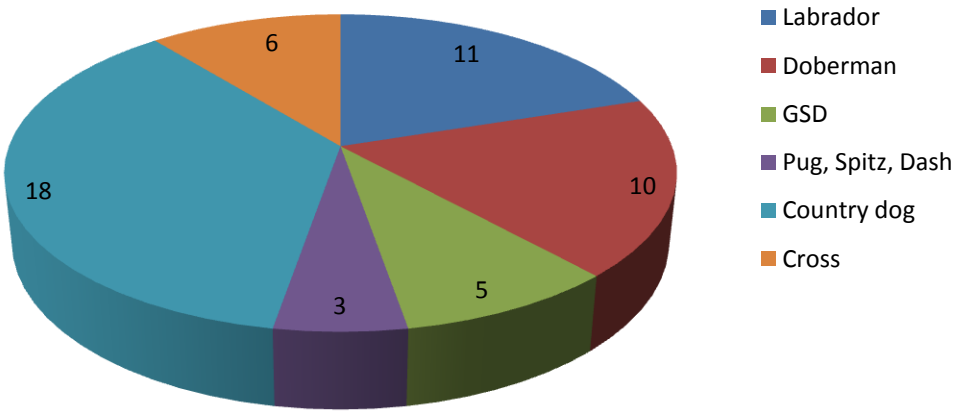
Figure 8 highlights the distribuion of subjects by their occupations.

Figure 8: Occupation wise distribution



The Pet owners 37 No.s (26%) were not included in this occupation wise distribution as they themselves belonged to a risk group. The Farmers 71 No.s (50%), Butchers 14 No.s (9.8%) and the Laboratory workers were 20 No.s (14%) in the present study. The numbers of dogs according to the different breeds are depicted in Figure 9. Pet dogs included in this study were grouped according to their breeds. The country dogs were more in number in this study.

Figure 9: Distribution of different breeds of dogs (n=53)



Their age was confirmed by the veterinarian. The age wise distributions of pet dogs are shown in Table 13.

Table 13: Age wise distribution of pet dogs (n= 53)

Breeds	< I yr	1-5 yrs	6-10 yrs	10-15 yrs	>15 yrs
Labrador	-	11	-	-	-
Doberman	-	8	1	1	-
German Shepherd	1	-	4	-	-
Pug, Spitz, Dash	1	2	-	-	-
Country dog	-	14	2	2	-
Cross dog	-	3	3	-	-

Table 14 highlights the risk of pet owners who have different period of exposure to their pets. Fifty five pet owners (78.57%) had exposure for 1 to 5 yrs, 12 no. (17.14%) for more than 5 yrs and 3 no.(4.28%) for less than a year.

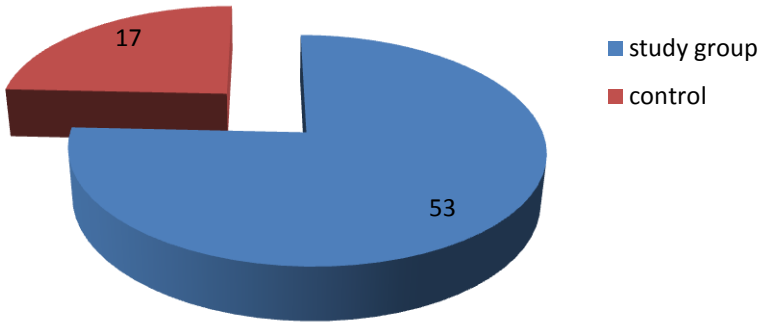
Table 14: Duration of contact in petowners (n= 70)

Duration of contact	< 1 yrs	1-5 yrs	> 5yrs
No. of Petowners	3	55	12
Percentage	4.28%	78.57%	17.14%

5.4. Blood culture for *leptospira*

Blood culture not performed with human samples. Among 70 pet dogs, 17 were taken as controls which were vaccinated regularly (Figure 10).

Figure 10: Distribution of Pet dogs

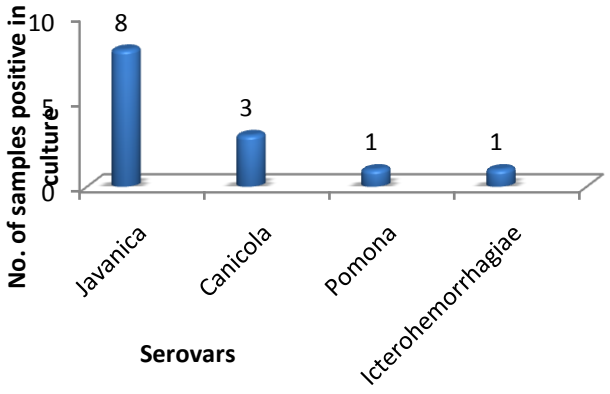


The leptospiral culture in EMJH semisolid medium was positive in 13 (24.5%) out of 53 pet dogs. Based on the serogrouping, 8 isolates were Javanica followed by Canicola 3 nos, Pomona and Icterohaemorrhagiae one each (Figure 11). The results of the leptospiral isolates were obtained from WHO collaborating centre for diagnosis, reference, research and training for leptospirosis, Andaman and Nicobar Islands are depicted in Table 15.

Table 15: Cross Absorption Agglutination Test (CAAT) results of Canine isolates

Isolates	Serogroup	Serovar	Strain
C-001	Pomona	Pomona	Pomona
C-003	Grippotyphosa	Grippotyphosa	Moskva V
C-005	Canicola	Canicola	Hond Uterecht IV
C-011	Javanica	Poi	Poi
C-014	Javanica	Poi	Poi
C-015	Javanica	Poi	Poi
C-024	Javanica	Poi	Poi
C-025	Javanica	Poi	Poi
C-030	Javanica	Poi	Poi
C-031	Canicola	Canicola	Hond Uterecht IV
C-036	Javanica	Poi	Poi
C-038	Canicola	Canicola	Hond Uterecht IV
C-061	Javanica	Poi	Poi

Figure 11: Serogroup distribution in Pet dogs by CAAT



5.5. SEROLOGY

5.5.1. Results of IgM ELISA:

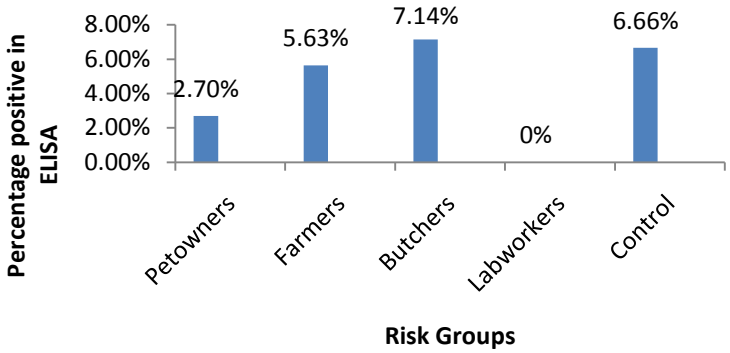
Out of 142 sera samples subjected for IgM ELISA (37 Pet owners, 71 farmers, 14 butchers and 20 laboratory workers), 6 were positive of which 4 (5.63%) were in farmers

followed by pet owners and butchers with one sample each. Out of 30 samples from healthy individuals included as controls, 2 were positive for anti leptospiral antibodies by ELISA. None of the laboratory workers included in this study showed positivity to ELISA. The serological results by ELISA were compared with that of Microscopic Agglutination Test (MAT). The results of IgM *leptospira* ELISA in relation to various risk groups and control are shown in Table 16 and Figure 12.

Table 16: Results of IgM *Leptospira* ELISA

Risk Groups	Total No.	No. of positive	Percentage
Pet owners	37	1	2.70%
Farmers	71	4	5.63%
Butchers	14	1	7.14%
Laboratory workers	20	-	-
Control	30	2	6.66%

Figure 12: Results of IgM *Leptospira* ELISA



5.5.2. Results of Microscopic Agglutination Test (MAT)

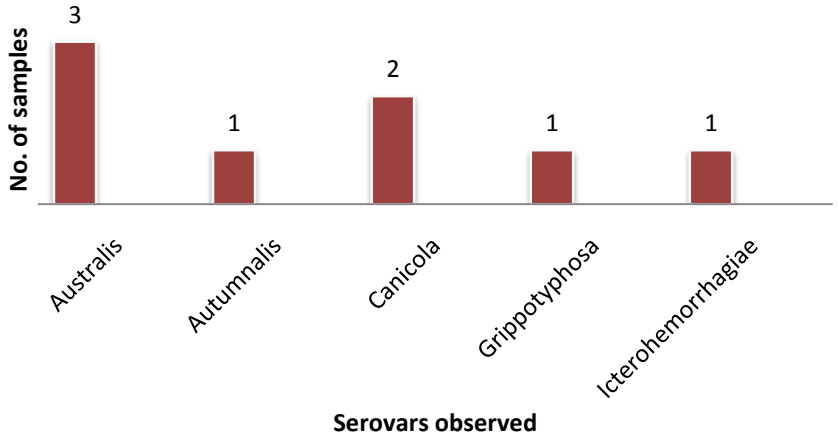
In this study, 172 sera from all human subjects and 70 pet dogs were included to analyze the presence of antibodies specific to Leptospiral serogroup. Serovars against which antibodies were observed by MAT were Australis, Autumnalis, Canicola, Grippotyphosa, Icterohemorrhagiae, Javanica, Pomona, Sejore.

5.5.2.1. MAT Results among human Subjects

5.5.2.1.1. MAT results in Pet owners' samples:

Out of 37 pet owner's sera subjected to MAT, 5 (13.5%) showed reactivity. Among the serovars, Australis predominated in 3 samples followed by Canicola (2 No.), Grippotyphosa, Icterohemorrhagiae and Autumnalis with one each (Figure 13).

Figure 13: MAT interpretation of Pet owners' samples



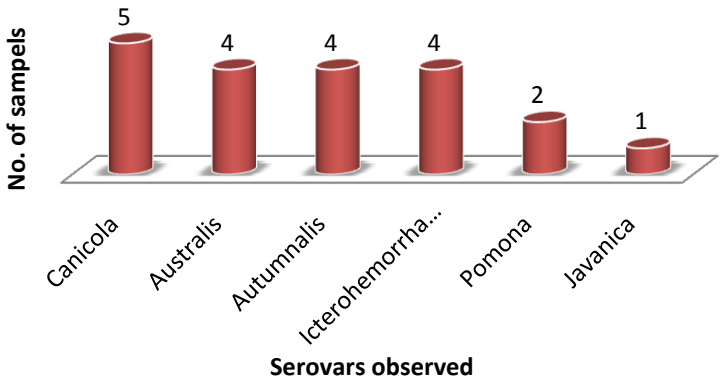
5.5.2.1.2. MAT results in farmers' samples:

Out of 71 farmers' samples seven showed reactivity (9.8%). Canicola dominated with reactivity in 5 samples with the highest titre of 1:1280 followed by Australis, Autumnalis and Icterohemorrhagiae each with 4 samples with the highest titre 1:640 among all serovars. One sample was reactive for Javanica and Sejroe at a titre of 1:80 each whereas 2 samples were reactive to Pomona at a titre of 1:640 (Table 17 and Figure 14).

Table 17: MAT results of farmers' samples

Serovar	No. of positives (n=7)	Highest titre value					
		1:80	1:160	1:320	1:640	1:1280	1:2560
Australis	4	1	-	-	3	-	-
Autumnalis	4	2	-	-	2	-	-
Canicola	5	2	-	1	1	1	-
Icterohemorrhagiae	4	1	-	2	1	-	-
Javanica	1	1	-	-	-	-	-
Pomona	2	-	-	-	2	-	-
Sejroe	1	1	-	-	-	-	-

Figure 14: MAT interpretation of farmers' samples

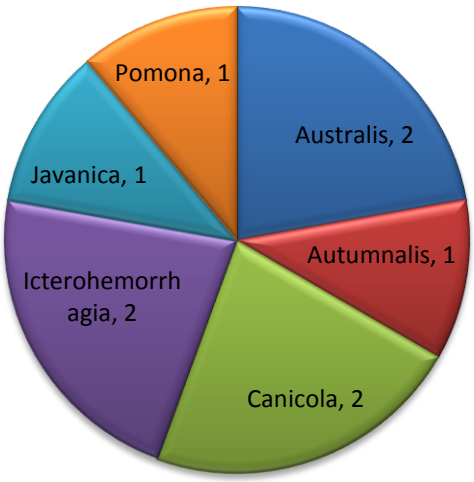


5.5.2.1.3. MAT results in butchers' samples:

Three (21.4%) samples, out of 14 showed MAT reactivity. Australis, Canicola, Icterohemorrhagiae dominated with reactivity of 2 samples each. The highest titre value was observed among Australis, Pomona with 1:1280. The serovar Canicola and

Icterohemorrhagiae reacted with highest titre of 1:640. Only one sample showed reactivity with Javanica at the highest titre of 1:320 (Figure 15).

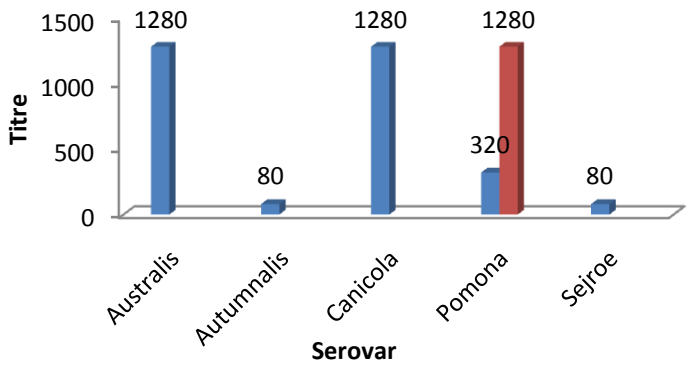
Figure 15: MAT interpretation of Butchers' samples



5.5.2.1.4. MAT results in Control samples:

A total of 30 healthy people who are not having history of leptospirosis were included as control. Among them, 2 (6.6%) samples showed MAT reactivity. The highest titre 1:1280 was observed against Australis, Canicola and Pomona. One sample was reactive for Pomona with 1:320 titre and 1 sample had mixed reactivity with Australis, Autumnalis, Canicola, Pomona and Sejroe with variation in titre. The numbers of samples showing MAT reactivity among control samples are depicted in figure 16.

Figure 16: MAT interpretation of control samples



All the values depicted in the figure are described as ratio eg. 1;1280, 1:80, 1:320

The overall seroprevalence among human subjects was tabulated (Table 18). The percentage of seroprevalence in pet owners, farmers, butchers and control were observed as 13.5%, 9.8%, 21.4% and 6.6% respectively. The overall reactivity in human samples was 10.8%. The laboratory workers who are repeatedly exposed to leptospiral culture and the principal investigator of this study showed non reactivity to both genus specific leptospiral ELISA and serovar specific MAT.

Table 18: MAT results of Human samples

Subjects	No. included (n=172)	No. of positives	Percentage
Pet owners	37	5	13.51%
Farmers	71	7	9.8%
Butchers	14	3	21.42%
Laboratory workers	20	-	-
Control	30	2	6.66%

5.5.2.2.1. MAT results among Pet Dogs

Among pet dogs, the highest titre of 1: 5120 was observed in 2 samples against *L. javanica.* Three samples showed a titre of 1:2560 (2 for Javanica and one for Canicola), 3 samples 1:160 titre (1 for Australis and 2 for Grippotyphosa). The serovar Javanica

dominated in 3 samples with a titre of 1:640. All the canine samples in the control group (n= 17) were negative for MAT. The number of samples showing serovars versus titre was tabulated. The total number of reactive sampled and the titre value for various canine samples and serogroup wise distribution is depicted in Table 19 and Table 20.

Table 19: MAT results of Canine samples

S. No	Sample reference No.	Serogroup	Titre
1.	C- 001	Pomona Australis grippotyphosa	1:1280 1:320 1:160
2.	C-003	Grippotyphosa Canicola Pomona Javanica Australis	1:1280 1:320 1:320 1:640 1:160
3.	C-005	Canicola	1:2560
4.	C-008	Canicola Icterohemorrhagiae Pomona	1:1280 1:1280 1:640
5.	C-011	Javanica Icterohemorrhagiae Pomona	1:2560 1:1280 1:640
6.	C-014	Javanica Pomona Grippotyphosa	1:2560 1:640 1:160
7.	C-015	Javanica Pomona	1:1280 1:320
8.	C-024	Javanica australis Pomona	1:5120 1:320 1:320
9.	C-025	Javanica Australis Pomona	1:1280 1:640 1:320
10.	C-030	Javanica	1:320
11.	C-031	Canicola Australis	1:640 1:320
12.	C-036	Javanica	1:5120

		Canicola Grippotyphosa	1:640 1:640
13.	C-038	Australis Canicola Javanica	1:1280 1:1280 1:320
14.	C-061	Javanica Canicola	1:640 1:320

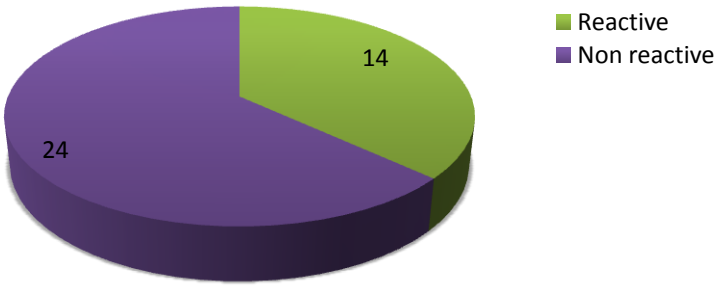
Table 20: MAT results of Canine samples - serovar and titre distribution

Serovar	No. of reactive samples (n=14)	Highest Titre Value					
		1:160	1:320	1:640	1:1280	1:2560	1:5120
Australis	6	1	3	1	1	-	-
Canicola	7	-	2	2	2	1	-
Grippotyphosa	4	2	-	1	1	-	-
Icterohemorrhagiae	2	-	-	-	2	-	-
Javanica	10	-	2	2	2	2	2
Pomona	8	-	4	3	1	-	-

5.5.2.2.2. Reactivity and Non reactivity of Index

A titre value of 1:80 and above is considered as positive in MAT (Natarajaseenivasan *et al.*, 2004; Ambiley *et al.*, 2013). In this study a total of 38 sera were reactive by MAT among 53 samples from pet dogs. Out of these 38 MAT reactive sera samples, 14 were considered as reactivity of index (titre value of 1:80 and above) and 24 sample, as non reactivity of index (titre value of less than 1:80 that is 1:40, 1:20) (Figure 17).

Figure 17: Samples supported reactivity and non reactivity of index



5.5.3. Comparison of ELISA and MAT:

In the diagnostic methods of leptospirosis, ELISA is considered as the prediction of positivity against genus level leptospires (Monalisa M et al., 2013). But MAT is the WHO reference test which confirms the leptospiral infection by using a battery of live Leptospiral antigens (WHO laboratory manual, 2007). The sensitivity and specificity of ELISA and MAT were well assessed by standardizing the ELISA reader at 460nm and inclusion of reactive titre of 1:80 and above in MAT respectively. The overall positivity of ELISA and MAT was observed as 5.1% and 10.8% respectively. Further, the positive reactivity results were well analyzed among individual risk groups and the percentages of seropositivity towards serological investigations were tabulated (Table 21).

Table 21: Comparative analysis of ELISA and MAT in human subjects

Groups	Total No.	No. of reactivity	
		ELISA	MAT
Pet owners	37	1 (2.70)	5 (13.51)
Farmers	71	4 (5.63)	7 (9.8)
Butchers	14	1 (7.14)	3 (21.42)
Laboratory workers	5	-	-
Control	30	2 (6.66)	2 (6.66)

[Figure in parenthesis denoted percentages]

Comparative analysis of culture and MAT among canine pets was performed by manual analysis and percentage description. All 13 culture positive samples were reactive in MAT. The Leptospiral cultures confirmed by CAAT showed predominance of Javanica serogroup among 8 samples followed by Canicola, Grippotyphosa, Pomona with 3, 1 and 1 sample respectively.

The MAT results highlighted that a maximum of 10 among 14 canine samples were identified as serovar Javanica. Two samples showed a titre value of 1:520. The percentage positivity of culture and MAT among the canine pets showed 24.6% and 26.4% respectively. The comparative analysis of culture and MAT among canine pets is shown in Table 22.

Table 22: Comparative analysis of Culture and MAT in canine pets

Pet dogs (n= 53)	Positive	Percentage	Predominant serovar
Culture	13	24.52	Javanica
MAT	14	26.41	Javanica

The overall comparative analysis of all the techniques included in this study was correlated (Table 23). It was observed that Australis, Canicola, Icterohemorrhagiae and Pomona dominated in human subjects and Javanica among pets.

Table 23: Comparative analysis of ELISA, MAT and Culture

Subjects	Total No. of subjects	ELISA	MAT	Culture
Human subjects				
Pet owners	37	1 (2.70)	5 (13.51)	ND
Farmers	71	4 (5.63)	7 (9.8)	ND
Butchers	14	1 (7.14)	3 (21.42)	ND
Laboratory workers	20	-	-	-
Control	30	2 (6.66)	2 (6.66)	ND
Animals				
Pet dogs	53	-	14 (26.41)	13 (24.52)

[Figure in the parenthesis denotes percentage]

5.5.4. Risk group wise serovar distribution

In pet owners, Autralis was the predominant serovar followed by Canicola. In farmers 5 out of 7 were having Canicola followed by Australis, Autumnalis and

Icterohemorrhagiae. In this study, butchers were found to be infected with multiple serovars. In control group, Pomona was the only serovar observed. The group wise serovar distribution is shown in Table 24.

Table 24: Groupwise serovar distribution

Groups	No. of Positive	Predominant serovar	Titre
Pet owners	5	Australis	1:80
Farmers	7	Canicola	1:80
Butchers	3	Australis Canicola Icterohemorrhagiae	1:80
Control	2	Pomona	1:320
Pet dogs	14	Javanica	1:320

The MAT results of pet owners and pet dogs were compared and depicted in the Table 25. Pet owner sample PO 1 and his dog sample C 001, both showed reactivity to serovars Australis and Grippotyphosa; additionaly Pomona was identified in dog's sample. Pet owner sample PO 3 had serovars Australis and Canicola which were also found in his pet dog C 003. In addition to that C 003 pet dog also had other serovars like Pomona, Javanica and Grippotyphosa. Pet owner PO 5 and his dog showed reactivity to serovar Canicola; additionaly pet owner PO 5 had serovars Pomona and Icterohemorrhagiae. Pet owners PO 14 and PO 17 did not have any correlation of serovars with their pet dogs. In this study 3 out of 5 pet owners' samples (60%) can be correlated by *Leptospiral* serovars with their respective pet dogs.

Table 25: Comparison of MAT results of pet owners and their pets

Reference	Pet owners	High titre	Reference	Pet dogs	High titre
PO 1	Australis Grippotyphosa	1:80 1:80	**C 001**	Australis Grippotyphosa Pomona	1:320 1:160 1:1280
PO 3	Australis Canicola	1:80 1:80	**C 003**	Australis Canicola Pomona Javanica Grippotyphosa	1:160 1:320 1:320 1:640 1:1280
PO 5	Pomona Icterohemorrhagiae Canicola	1:80 1:80 1:80	**C 005**	Canicola	1:2560
PO 14	Autumnalis	1:80	**C 014**	Grippotyphosa Pomona Javanica	1:640 1:640 1:2560
PO 17	Australis	1:80	**C 017**	Javanica Pomona	1:320 1:320

Even though it was not confirmed by molecular studies like Polymerase Chain Reaction and In situ hybridisation, 60% correlation have been noted in serovars between pet owners and their pets.

DISCUSSION

6.0. DISCUSSION

The main aim of the study was to assess the seroprevalence of Leptopsirosis among human risk groups (n=142); canine pet owners (37), agricultural workers (71), butchers (14) and laboratory workers (20) in and around Thiruchirapalli, Tamil Nadu, India. Leptospirosis is a common spirochetal zoonotic disease of worldwide distribution. It is considered as an occupational disease of persons engaged in agriculture, animal slaughtering, laboratory works and forestry. As per WHO/ Leptospirosis burden Epidemiology Reference Group (LERG), it is estimated that 0.1 to 1 per 100,000 people living in temperate climates are affected each year, with the number increasing to 10 or more per 100,000 people living in tropical climates. Country wide incidence of Leptospirosis occording to WHO/ LERG is depicted in table 26.

Table 26: Country wide incidence of Leptospirosis[128]

WHO region	Median incidence of Leptospirosis 100,000 persons
Africa	95.5
Eatern Mediterrarian	-
Europe	0.5
America	12.5
South East Asia	4.8
Western Pacific	66.4
World	5.1

6.1. Age wise distribution:

Age wise analysis showed that 80 out of 172 (46.5%) subjects comes under 31-45 years followed by 45 (26.1%) in the age group of 16-30 years. This is in concordance with other studies, which also showed the same age distribution. Leptospirosis was less

frequent in children <15 yrs of age and older adults >75 yrs of age because they are having limited infectious exposure[64]. Few studies showed that children in the age group of 7-14 yrs had serological evidence of *leptospiral* infection due to playing in stagnant rain water. The mean seropositivity was found to be more in patients of >20 yrs in both sexes (Koteeswaran *et al.,* 2006). In a study, out of 216 cases 19 cases were found to be in the age group of 0-5 yrs, 13 between 5-15 yrs and 45 cases were adults and it is depicted in table 27; this type of age wise distribution was observed in most of the studies[23].

Table 27: Age wise distribution of seroprevalence of Leptospirosis by Monalisa *et al*., 2013 (n= 216)

Age group	0-5 yrs	5-15 yrs	>15 yr
Cases	19	13	45
Percentage positive	8.79%	6.01%	20.83%

6.2. Sex wise distribution:

Epidemiological data (age, sex, occupation and duration of contact with pet dogs) collected from the study subjects showed that Leptospiral seroprevalence was 82.35% in males and 17.64% in females. In our study, males dominated over females. This is consistent with other studies which also showed a male preponderance. In a study performed in North India showed that male patients (49, 57%) outnumbered female patients (39, 43%) [79]. A study conducted in West Bengal showed a male: female ratio of 7:4 [33]. This high prevalence in males when compared to females is due to continuous exposure of males to risk environment. The infection mostly affects males and this sex difference was noted in most of the studies[10] [102] [127]. This is usually attributed to occupation and behavioral factors and it tends to vanish if both sexes are given equal

exposure [128] [90]. The major reason for this is, most of the men are out door workers compared to women particularly involved in agriculture and sewage cleaning. In our study, butcher were only males and among pet owners (29/37, 78.37%) also males dominated. In farmers, male and female constituted 49.2% and 50.7% respectively. A clinical and serological study on Leptospirosis from hospital based observational study at CMC, Vellore revealed equal distribution of male and female [22], and this may be due to nature of referral cases as CMC Vellore is a tertiary care hospital.

6.4.0. Seroprevalence:

The overall seroprevalence of the asymptomatic subjects in this study was 9.8%. Studies from different parts of India showed seroprevalence ranging from 17.8% to 40.5%. In 2013, a seroprevalence of 35.64% was reported from West Bengal. This is in discordance with the present study which showed low seroprevalence. This may be due to difference in the inclusion of study subject. In the above mentioned studies febrile patients were included but the present study included asymptomatic human subjects only who are at risk of Leptospirosis.

6.4.1. Seroprevalence among occupational risk groups:

Along with farmers, pet owners, butchers and laboratory workers were included to understand the *leptospiral* epidemiology among risk groups. Risk activities observed in this study are close contact with pet dogs, agricultural practices like sowing and harvesting, animal slaughtering and handling carcasses and laboratory practices. As per

the data obtained, most of the subjects included in this study are prone to Leptospirosis. In the present study, the seroprevalence of Leptospirosis among pet owners was 13.5%, among farmers and butchers it was 9.8% and 21.4% respectively. It is depicted in the table 28.

Table 28: Seroprevalence in risk groups

Risk group (Nos)	No. of positives	Percentage
Pet owners (37)	5	13.5%
Butchers (14)	3	21.4%
Farmers (71)	7	9.8%

Agricultural practices are recognized as an important occupational hazard [78] and the same was observed in this study by including 71 farmers (55.9%). Seroprevalence studies among occupational groups such as slaughter house workers [90 112], agricultural workers of Tamil Nadu have shown 80% and 17.6% respectively[23]. Low prevalence in butchers in this study may be due to inclusion of less number of samples in this group.

6.4.2. Risk activities observed in this study:

Studies have shown that the occupation which has the maximum risk of exposure was out door manual workers with 39.4% positivity[46]. Major epidemiological risk factors noted in a study conducted in North India and West Bengal include wet environmental living conditions, lack of personal protective equipment using practices, infestation of indwelling with rodents, bare foot walking in farm lands and contact with animals[127].

In the present study, history of bare foot walking in farm lands (40%) and infestation of indwelling with rodents (25%) has been elicited in farmers group.

6.5. Canine pets inclusion in the study:

In this study a total of 70 canine subjects were included (Case 53, Control 17). In general the dogs were compiled into 3 breed categories like small breeds (Pug, Spitz, Dash etc), large breeds (Labrodar, Doberman, German sheperd), terrier breeds (Country dogs, Cross dogs) which were included in our study for investigating *leptospiral* seroprevalence. There is an anecdotal perception among veterinarians that urban dogs have less exposure to Leptospirosis than the rural dogs[86]. In the present study all the dogs included were from urban areas. Out of 70 pet dogs, 17 had the history of regular annual vaccination for Leptospirosis which were taken as control and all were non reactive serologically. In the study group, out of 53 canine pets, 30 had the history of irregular vaccination and 23 had no history of vaccination which is depicted in the table 29.

Table 29: Vaccination profile of canine pets (n=70)

vaccination	Total No.s Percentage %)	No. of Positive (percentage %)
Regularly vaccinated	17(24.28)	-
Irregularly vaccinated	30(42.85)	6 (20)
Unvaccinated	23(32.85)	8 (34.78)

Seventy eight percentage of pet owners gave history of exposure for 1-5 years duration. The seroprevalence of *leptospiral* antibodies in canine pets was (14/53) 26.4%. It is in concordance with the study conducted in Namakkal, Tamil Nadu. In the year 2013, in Namakkal, Tamil Nadu, sera samples of dogs belonging to vaccinated, unvaccinated-semiowned, and stray dogs showed seroprevalence of 57%, 28.8% and 35.2% respectively[86]. It also indicated that multiple serovars are known to be circulating in the local canine population. A study performed in Kerala showed seroprevalence of 71.12% in canines which included vaccinated dogs and healthy unvaccinated dogs and

dogs with suspected leptospirosis[38]. Yet another study has emphasized that even vaccinated dogs can get infection with multiple *leptospiral* serovars[86]. Thus both vaccinated and unvaccinated dogs are considered as risk in transmission of leptospires. The observation of Leptospirosis among pet dogs has increased due to contact with wild and stray dogs. The pet owners are infected accidentally while exposed to dog's urine in the environment or while handling the animals. Based on the serology and culture prevalence of Leptospirosis in pet dogs and pet owners, from the study, it is likely that pet owners are more prone for Leptospirosis. A cross sectional study carried out in Switzerland in 2011, included pet owners' of the affected dogs and veterinarians for seroreactivity to *Leptospira* serovars. Eventhough all human subjects included in the study were seronegative for *Leptospira* serovars, they emphasizes the risk level is undoubtedly the hygienic measures they adhered to[84].

In Thiruchirapalli area, according to our knowledge this is the first study on canine pets. No comparative analysis could be made out among pet dogs and its owners due to lack of literature. A study showed dogs aged 5 yrs or older had a significant reduced titre to *leptospiral* serovars when compared to the dogs of <5 yrs of age (Muller *et al.*, 2011).

6.5.1. Culture and serovar determination:

The cultures of leptospires were examined for signs of growth either by turbidity or by a ring of growth (Dingers ring); confirmed by using dark field illumination from day 2 up to 6 weeks[90]. In the present study, all the isolates (13/53) were positive by dark field microscopy as described in previous studies[90]. In most cases, Dark Field Microscopy (DFM) is not much recommended due to false positive and false negative

results[9]. The typical motility of the leptospires in dark field microscopy when correlated with environmental and clinical parameters may aid in early diagnosis. Care was taken to exclude artefacts like lysed RBCs, fibrils, slide cracks, dust threads which can be mistaken for leptospires. According to Cross Absorption Agglutination Test for cultures, the serogroup Javanica dominated in 8 blood cultures followed by Canicola with 3, Grippotyphosa and Pomona with 1 each. This study is the first to document the prevalence of leptospires and its antibodies in unvaccinated and irregularly vaccinated pet dogs in comparison to vaccinated pet dogs in the Cauvery river valley (Thiruchirapalli, Tamil Nadu, INDIA, Southest Asia). The previous report in this area was found positive to *leptospiral* isolates among dairy cattles (87%) experiencing a high degree of abortion, infertility and still birth[6].

6.6.0. Comparative analysis of serological tests:

6.6.1. Enzyme Linked Immuno Sorbent Assay (ELISA):

Enzyme Linked Immuno Sorbent Assay is the most widely used reliable test for diagnosing leptospirosis[127]. IgM ELISA was the best choice in many studies regardless of the stage of diseas. Moreover, these IgM antibodies can persist for more than 12 months and longer in contrast to IgG antibodies, which can be detected only up to 3 months in majority of cases[113]. So, anti IgM ELISA test is a suitable method for detecting leptospiral antibodies in human sera for diagnostic and epidemiological purposes[40]. In this study, Pan Bio IgM ELISA kit was used in all human blood samples. Serum samples from apparently healthy human subjects belonging to risk groups

including pet dog owners, farmers, butchers and laboratory workers were screened. Among them 8 out of 142 (5.63%) showed positivity in ELISA. A clinico epidemiological study conducted in north India showed an increased incidence of Leptospirosis from 11.7% in 2004 to 20.5% in 2008[127]. In a study conducted in West Bengal, IgM ELISA positivity was 36.27% [33]. A comparative study in 2015 showed IgM ELISA seropositivity of 46% with positive predictive value of 8.7% [129]. Even though the percentage of the positivity is low, the risk of exposure to infection is high due to continuous shedding of leptospires even after recovery from the infection. Seroprevalence of Leptospirosis was 56.97% in Irulas. They were generally designated as "Rat catchers" and may be the reason for higher prevalence in this community[80].

6.6.2. Microscopic Agglutination Test (MAT):

The results of MAT among human risk groups were observed as 9.8%, where farmers predominated with 41.2%. The pet owners showed seroreactivity of 29.4% followed by butchers 17.6%. The predominant serovar was Australis among pet owners with the highest titre of 1:80 in 3 samples. Among the farmers group, Canicola dominated with the highest titre of 1:1280. Two samples were observed with the highest titre of 1:1280 among butchers where the serovars Australis and Pomona dominated. The serovar Canicola and Icterohemorrhagiae were also found among 2 samples with high titre of 1:640. Rarely, Javanica was identified among human subjects especially in butchers with 1:320 titre values.

In the present study, the predominant serovar was Australis in both pet owner and butcher group. This is comparable with other studies which also showed Australis as a

prevalent serovar in humans. The predominant serogroup among humans was identified as Australis during 2004- 2006 based on the MAT and conclusive data obtained by CAAT confirmation and serotyping[23]. A report from Kerala showed prevalence of serovar Australis followed by Pomona[131]. But two other studies showed serovar Autumnalis as a predominant type. In a study performed in dairy farm workers in Thiruchirapalli showed Autumnalis as a predominant serovar[6]. A study in Vellore also showed Autumnalis as a predominant serovar in febrile patients[22]. This discordance may be due to multiple serovar infection existing in endemic areas. Moreover, paradoxical reactions and cross reactions are quite common in MAT which can interfere with the identification of infecting serovar[29]. Table 30 shows a comparative evaluation of predominant serovar in studies conducted in India.

Table 30: Comparative evaluation of predominant serovar in various studies in India

Author	Place	Journal /yr	Risk group	Predominant serovar
Soman et al	Kerala	Journal of agricultur and veterinary science/ 2014 Veterinary world 2014; Oct: 759-764.	Suspected cases	Australis
Natarajaseenivasan et al	Thiruchirapalli, Tamil Nadu	Southeast Asian journal of tropical medicine and public health/ 2011 Southeast Asian J Trop Med Pub Hlth 2011; 42: 679-686.	Dairy farm workers	Autumnalis
Vimala et al	CMC, Vellore,	International journal	Febrile cases	Autumnalis

	Tamil Nadu	of Microbiologist/ 2014 Int J Microbiol 2014; 2: 344-348.		

Among the pet dogs included in the study serovar Javanica dominated in 10 samples with the highest titre of 1:5120 in 2 samples. The serovar Javanica was equally distributed with 2 samples each in 1:320, 1:640, 1:1280, 1: 2560 and 1:5120 titre. This is comparable with the other studies performed on stray dogs in different parts of the country. In Madurai (Tamil Nadu), 46% positivity of Javanica was identified among dogs followed by Pyrogenes [15]. In Chennai, Pyrogenes was identified as dominating serovar among dogs[23] whereas it is Autumnalis in a study conducted in Kerala [15]. In the present study, the prevalent serovar found in dogs was Javanica. Interestingly, most of the studies conducted previously have not reported Javanica as a predominant serovar in pet dogs. It has been speculated that the serovar dominating in the present study is present in the rodent in the environment and further the dogs might have been exposed to the same environment. Further studies are needed to confirm this observation.

Sometimes, serovar determination by MAT among pet dogs and its owners are not correlated mainly due to low positive predictive value (PPV). The reason for this low PPV could be due to the various diagnostic pitfalls of MAT including,

1. Antibodies may not be detectable when the causative strain is not represented in the panel of antigens.
2. Only a low titre is found with serovar which is not considered as positive.
3. Never possible to be sure that the panel is complete since new unidentified leptospires may cause the disease [129].

To determine this, high profile CAAT with mixed or multiple serovars as control to predict or identify the newer serovars was used. In this study, the pet owners' serovars are comparable with their respective pet dogs and 3 out of 5 samples of pet owners', correlate with their pet dog sample based on serovar. Though further research is needed in molecular level to confirm the relatedness of strains between the two groups (pet owners and their pet dogs), the present study emphasizes the possible role of transmission of Leptospirosis from the canine pets to their owners. By our stud it can be said that the pet dogs are one of the major responsible factors for the transmission of infection to its owners. It also provides a better understanding of the role of these animals in transmission dynamics and epidemiology of human Leptospirosis in a particular area.

6.6.3. Comparative analysis of ELISA and MAT:

In this study, for human subjects, both ELISA and MAT were performed. The overall seropositivity by ELISA was 4.6% (8/172) and MAT reactivity was 9.8% (17/172). Even though ELISA is a genus specific test, the seropositivity is low compared to MAT results in this study. It is because, ELISA used in this study can identify only IgM antibodies. But MAT can identify both IgM and IgG antibodies. Moreover, the study subjects were asymptomatic apparently healthy human subjects, in whom the IgM response may be low. The MAT with its unsurpassed sensitivity and specificity is the gold standard in the diagnosis of Leptospirosis[47]. Unfortunately, the test is difficult to standardize and has intrinsic limitation due to subjective interpretation of results [39]. The low specificity of serological results derived from cross reactivity between different serovars belonging to same serogroups making the interpretation difficult.

6.7. Comparative analysis of culture and serological tests:

The comparative analysis of *leptospiral* cultures and serological tests provide viable path to determine the stage of infection and presence of spirochetes. In the present study, culture was done for dog samples and results were compared with MAT. Culture positivity confirmed by Dark Field Microscopy (DFM) and Cross Absorption Agglutination Test (CAAT) was 24.52%. The MAT reactivity was 26.41% slightly higher than culture positivity. Some studies have emphasized that definitive diagnosis is culturing and isolating the leptospires which also identifies the infecting serovars[32]. But a recent study has shown that DFM and culture has limited value in diagnosing Leptopsirosis with serology being the mainstay[129].

6.8. Serovar prevalence:

In the present study, most of the positive sera tested by MAT had antibodies against Javanica. In some samples 2 or more serovars (ie) Australis, Autumnalis, Icterohemorrahgiae, Canicola and Javanica showed reactivity. This could be due to –

1. The antibodies cross react with other serovars.

2. Heterologus cross reactions with other serovars. Occasionally the heterologus reaction may be positive and homologus reaction may be negative and this reaction may be called paradoxical reaction.

3. Simultaneous infection with multiple serovars[129].

In tropical areas like Thiruchirapalli, the risk of Leptospirosis is found crucial, thus health education is of paramount importance and should stress the need to apply all the preventive measures relevant to risk activities. This can be achieved by creating

awareness about Leptospirosis among risk groups especially canine pet owners, implementation of proper laboratory diagnostics and notification systems preferentially associated with surveillance.

Strength of study: 1. Leptospiral serovar correlation has been carried out between pet owners and their canine pets – probably first of its kind in India to our knowledge. 2. Cross Absorption Agglutination Test (CAAT) has been performed, which is done only in very few centres for serovar and strain confirmation.

Limitation : 1. It is a single center study. 2. Blood culture not done for human subjects due to low yield.

Future area of work: 1. Identifying new serovars by PCR techniques and incorporating them as antigen in serological kits. 2. Transmission dynamics related work.

SUMMARY

7. SUMMARY

- A prospective cross-sectional observational study was undertaken between April 2014 to March 2015 to identify the presence of Leptospiral antibodies in 142 subjects (95 males and 77 females), in and around Thiruchirapalli, Tamilnadu, INDIA. Fifty three dogs of age 2 months to 15 years were included in the study to screen for the presence of leptospires and its specific antibodies.
- The seroprevalence of the asymptomatic study subjects in this study were 9.8%. The predominant serovar was Australis in both pet owner and butcher group and Canicola in farmers.
- In Cross Absorption Agglutination Test (CAAT) for blood culture positive (24.5%) pet dogs' samples, the serogroup Javanica dominated followed by Canicola, Grippotyphosa and Pomona. This study shows the prevalence of leptospires and its antibodies in unvaccinated and irregularly vaccinated pet dogs in comparison to regularly vaccinated pet dogs in Thiruchirapalli, Tamil Nadu, INDIA.
- Overall seropositivity for human subjects by ELISA was 4.6% (8/172) and MAT reactivity was 9.8% (17/172).
- The present study emphasizes the possible role of transmission of Leptospirosis from the canine pets to their owners as, 60% correlation observed between pet owners' and their pet dogs based on serovar.

CONCLUSION

8. CONCLUSION

Leptospirosis is a multi prong disease with various challenges for both clinicians and laboratory physicians. Its prevalence is more even in asymptomatic subjects with history of risk factor association. It causes increased morbidity and mortality in susceptible individuals. Although the results obtained in this study may not be considered conclusive, it emphasises the need of incorporating new emerging serovars in the vaccine against Leptospirosis for pet animals and the possible mode of spread by close contact and irregular vaccination practices among pet owners.

In Thiruchirapalli, the seroprevalence and culture positivity of leptospires among the risk groups and the animals indicates the need for increased awareness and knowledge about the disease severity and suspicion of Leptospirosis among clinicians which will help to reduce morbidity and mortality associated with the disease.

ANNEXURE I

MASTER CHART

S.no	Sample Code	Age	Sex	Date of Collection	Risk Group	Duration of contact in years	IgM ELISA report	MAT report	MAT serovar report
1	1	36	M	31.1.15	petowner	2	pos	pos	aut,grip
2	2	42	F	31.1.15	petowner	4	neg	neg	-
3	3	55	M	31.1.15	petowner	1	neg	pos	aus,can
4	4	40	F	31.1.15	petowner	3	neg	neg	-
5	5	46	M	31.1.15	petowner	5	neg	pos	ict,poM,
6	6	27	M	2.2.15	petowner	8	neg	neg	-
7	7	38	M	2.2.15	petowner	2	neg	neg	-
8	8	52	F	2.2.15	petowner	1	neg	neg	-
9	9	70	M	2.2.15	petowner	2	neg	neg	-
10	10	43	M	2.2.15	petowner	1	neg	neg	-
11	11	57	M	11.2.15	petowner	1	neg	neg	-
12	12	40	M	11.2.15	petowner	2	neg	neg	-
13	13	30	M	11.2.15	petowner	5	neg	neg	-
14	14	24	M	17.2.15	petowner	4	neg	pos	aut
15	15	52	M	17.2.15	petowner	3	neg	neg	-
16	16	42	M	17.2.15	petowner	1	neg	neg	-
17	17	35	M	17.2.15	petowner	8	neg	pos	aus
18	18	42	M	17.2.15	petowner	6	neg	neg	-
19	19	29	M	17.2.15	petowner	5	neg	neg	-
20	20	34	F	23.2.15	petowner	4	neg	neg	-
21	21	39	F	23.2.15	petowner	3	neg	neg	-
22	22	42	M	23.2.15	petowner	4	neg	neg	-
23	23	40	M	23.2.15	petowner	5	neg	neg	-
24	24	35	M	23.2.15	petowner	1	neg	neg	-
25	25	24	F	23.2.15	petowner	8	neg	neg	-
26	26	30	M	23.2.15	petowner	2	neg	neg	-
27	27	40	M	23.2.15	petowner	1	neg	neg	-
28	28	37	F	23.2.15	petowner	1	neg	neg	-
29	29	43	F	23.2.15	petowner	2	neg	neg	-
30	30	28	F	23.2.15	petowner	3	neg	neg	-
31	31	40	M	23.2.15	petowner	1	neg	neg	-
32	32	52	M	23.2.15	petowner	4	neg	neg	-
33	33	38	F	23.2.15	petowner	3	neg	neg	-
34	34	39	M	23.2.15	petowner	5	neg	neg	-
35	35	32	M	23.2.15	petowner	2	neg	neg	-
36	36	45	F	23.2.15	petowner	2	neg	neg	-
37	37	40	M	23.2.15	petowner	4	neg	neg	-

38	1	53	M	1.12.14	FarMer	31	neg	pos	can,aut
S.no	**Sample**	**Age**	**Sex**	**Date oF Collection**	**Risk Group**	**Duration of contact in years**	**IgM ELISA report**	**MAT report**	**MAT serovar report**
39	2	63	M	28.2.15	FarMer	43	neg	neg	-
40	3	55	M	29.2.15	FarMer	28	neg	pos	can,,aut
41	4	45	M	29.2.15	FarMer	22	neg	neg	-
42	5	70	M	29.2.15	FarMer	54	neg	neg	-
43	6	70	M	29.2.15	FarMer	43	neg	neg	-
44	7	25	M	29.2.15	FarMer	8	neg	pos	ict,aus
45	8	51	F	2.3.15	FarMer	32	neg	neg	-
46	9	35	F	2.3.15	FarMer	15	neg	neg	-
47	10	35	M	2.3.15	FarMer	17	neg	neg	-
48	11	45	F	2.3.15	FarMer	26	neg	neg	-
49	12	33	M	2.3.15	FarMer	12	neg	neg	-
50	13	65	M	2.3.15	FarMer	48	pos	pos	aus,grip
51	14	65	M	2.3.15	FarMer	43	neg	neg	-
52	15	24	F	2.3.15	FarMer	7	pos	pos	aus,aut
53	16	54	M	2.3.15	FarMer	26	neg	neg	-
54	17	60	F	2.3.15	FarMer	42	neg	neg	-
55	18	40	F	2.3.15	FarMer	25	neg	neg	-
56	19	57	F	2.3.15	FarMer	30	neg	neg	-
57	20	41	F	2.3.15	FarMer	15	neg	neg	-
58	21	41	F	2.3.15	FarMer	17	neg	neg	-
59	22	48	F	3.3.15	FarMer	25	neg	neg	-
60	23	60	F	3.3.15	FarMer	35	neg	neg	-
61	24	28	F	3.3.15	FarMer	10	neg	neg	-
62	25	41	F	3.3.15	FarMer	24	neg	neg	-
63	26	70	F	3.3.15	FarMer	55	neg	neg	-
64	27	46	M	5.3.15	FarMer	25	neg	neg	-
65	28	45	F	5.3.15	FarMer	20	neg	neg	-
66	29	63	F	5.3.15	FarMer	40	neg	neg	-
67	30	35	F	5.3.15	FarMer	15	neg	neg	-
68	31	45	F	7.3.15	FarMer	20	neg	neg	-
69	32	47	F	7.3.15	FarMer	25	neg	neg	-
70	33	60	M	7.3.15	FarMer	35	neg	neg	-
71	34	60	F	7.3.15	FarMer	30	neg	neg	-
72	35	47	F	7.3.15	FarMer	27	neg	neg	-
73	36	69	M	9.3.15	FarMer	50	neg	neg	-
74	37	45	F	9.3.15	FarMer	25	neg	neg	-

75	38	37	F	9.3.15	FarMer	15	neg	neg	-
76	39	40	F	9.3.15	FarMer	20	neg	neg	-
S.no	**Sample**	**Age**	**Sex**	**Date oF Collection**	**Risk Group**	**Duration of contact in years**	**IgM ELISA report**	**MAT report**	**MAT serovar report**
77	40	42	M	9.3.15	FarMer	24	neg	neg	-
78	41	45	F	18.3.15	FarMer	25	neg	neg	-
79	42	60	M	18.3.15	FarMer	40	neg	neg	-
80	43	44	M	18.3.15	FarMer	21	neg	neg	-
81	44	64	F	18.3.15	FarMer	45	neg	neg	-
82	45	40	F	18.3.15	FarMer	20	neg	neg	-
83	46	50	F	18.3.15	FarMer	25	neg	neg	-
84	47	65	M	18.3.15	FarMer	47	neg	neg	-
85	48	75	F	18.3.15	FarMer	56	neg	neg	-
86	49	45	M	18.3.15	FarMer	20	neg	neg	-
87	50	73	M	19.3.15	FarMer	50	neg	neg	-
88	51	55	F	19.3.15	FarMer	25	neg	neg	-
89	52	28	F	19.3.15	FarMer	10	neg	neg	-
90	53	50	F	19.3.15	FarMer	25	neg	neg	-
91	54	70	F	19.3.15	FarMer	52	neg	neg	-
92	55	51	F	19.3.15	FarMer	25	neg	neg	-
93	56	48	M	19.3.15	FarMer	24	neg	neg	-
94	57	48	M	19.3.15	FarMer	23	neg	neg	-
95	58	21	M	19.3.15	FarMer	3	neg	neg	-
96	59	57	M	19.3.15	FarMer	32	neg	neg	-
97	60	60	M	19.3.15	FarMer	40	neg	neg	-
98	61	50	M	19.3.15	FarMer	25	neg	neg	-
99	62	45	F	19.3.15	FarMer	20	neg	neg	-
100	63	40	M	19.3.15	FarMer	20	neg	neg	-
101	64	62	M	20.3.15	FarMer	43	neg	neg	-
102	65	48	M	20.3.15	FarMer	24	neg	neg	-
103	66	59	M	20.3.15	FarMer	34	neg	neg	-
104	67	45	M	20.3.15	FarMer	27	neg	neg	-
105	68	35	M	20.3.15	FarMer	10	pos	pos	can,aus
106	69	43	F	20.3.15	FarMer	22	neg	neg	-
107	70	25	F	20.3.15	FarMer	3	neg	neg	-
108	71	51	F	20.3.15	FarMer	23	neg	neg	-
109	72	64	F	20.3.15	FarMer	45	neg	neg	-

110	1	40	M	17.4.15	butcher	20	neg	pos	aut,ict
111	2	27	M	17.4.15	butcher	8	neg	neg	-
112	3	22	M	17.4.15	butcher	3	neg	neg	-
113	4	30	M	17.4.15	butcher	10	neg	neg	-
S.no	**Sample**	**Age**	**Sex**	**Date oF Collection**	**Risk Group**	**Duration of contact in years**	**IgM ELISA report**	**MAT report**	**MAT serovar report**
114	5	30	M	17.4.15	butcher	12	neg	pos	can,aut
115	6	39	M	17.4.15	butcher	20	neg	neg	-
116	7	40	M	17.4.15	butcher	22	pos	pos	aus,poM,
117	8	48	M	17.4.15	butcher	25	neg	neg	-
118	9	30	M	17.4.15	butcher	12	neg	neg	-
119	10	42	M	17.4.15	butcher	22	neg	neg	-
120	11	32	M	17.4.15	butcher	15	neg	neg	-
121	12	43	M	17.4.15	butcher	20	neg	neg	-
122	13	40	M	17.4.15	butcher	18	neg	neg	-
123	14	48	M	17.4.15	butcher	23	neg	neg	-
124	15	40	M	17.4.15	butcher	20	neg	neg	-
125	16	22	M	17.4.15	butcher	4	neg	neg	-
126	17	28	M	17.4.15	butcher	10	neg	neg	-
127	18	46	M	17.4.15	butcher	26	neg	neg	-
128	19	39	M	17.4.15	butcher	22	neg	neg	-
129	20	33	M	17.4.15	butcher	15	neg	neg	-
130	21	62	M	17.4.15	butcher	40	pos	pos	-
131	22	54	M	17.4.15	butcher	25	neg	neg	-
132	23	32	M	17.4.15	butcher	15	neg	neg	-
133	24	34	M	17.4.15	butcher	16	neg	neg	-
134	25	36	M	17.4.15	butcher	14	neg	neg	-
135	26	25	M	17.4.15	butcher	8	neg	neg	-
136	27	32	M	17.4.15	butcher	17	neg	neg	-
137	28	45	M	17.4.15	butcher	23	neg	neg	-
138	1	25	F	6.5.15	control	2	neg	neg	-
139	2	47	M	6.5.15	control	8	neg	neg	-
140	3	30	F	6.5.15	control	3	neg	neg	-
141	4	32	F	6.5.15	control	1	neg	neg	-
142	5	40	M	6.5.15	control	4	neg	neg	-
143	6	32	F	6.5.15	control	3	neg	neg	-
144	7	30	F	6.5.15	control	5	neg	neg	-
145	8	28	F	6.5.15	control	2	neg	neg	-

146	9	40	F	6.5.15	control	3	neg	neg	-
147	10	28	F	6.5.15	control	4	neg	neg	-
148	11	26	F	6.5.15	control	2	neg	neg	-
149	12	28	F	6.5.15	control	3	neg	neg	-
150	13	40	F	6.5.15	control	7	neg	neg	-
151	14	28	F	6.5.15	control	2	neg	neg	-
S.no	**Sample**	**Age**	**Sex**	**Date oF Collection**	**Risk Group**	**Duration of contact in years**	**IgM ELISA report**	**MAT report**	**MAT serovar report**
152	15	35	M	6.5.15	control	6	neg	neg	-
153	16	40	F	6.5.15	control	5	neg	neg	-
154	17	25	M	6.5.15	control	1	neg	neg	-
155	18	25	M	6.5.15	control	1	neg	neg	-
156	19	25	M	6.5.15	control	1	neg	neg	-
157	20	25	M	6.5.15	control	1	neg	neg	-
158	21	24	F	6.5.15	control	1	neg	neg	-
159	22	24	F	6.5.15	control	1	neg	neg	-
161	23	35	F	6.5.15	control	5	neg	neg	-
162	24	25	F	6.5.15	control	2	pos	pos	aus,poM
163	25	25	F	6.5.15	control	1	neg	neg	-
164	26	25	F	6.5.15	control	1	pos	pos	can,poM
165	28	35	F	6.5.15	control	7	neg	neg	-
166	29	24	F	6.5.15	control	4	neg	neg	-
167	30	30	F	6.5.15	control	2	neg	neg	-
168	31	35	F	6.5.15	control	3	neg	neg	-
169	32	30	F	6.5.15	control	2	neg	neg	-
170	33	24	F	6.5.15	control	1	neg	neg	-
171	34	30	F	6.5.15	control	2	neg	neg	-
172	35	60	M	6.5.15	control	25	neg	neg	-

PROFORMA

SAMPLE NO: LAB NO:

NAME OF THE PATIENT:

AGE/SEX:

EDUCATIONAL STATUS: LITERATE/ ILLITERATE

RESIDENTIAL ADDRESS:

MOBILE NO:

OCCUPATION:

PAST ILLNESS IF ANY:

DURATION OF FEBRILE ILLNESS: TREATMENT HISTORY: IF YES-

DRUGS TAKEN

(PROLONGED FEVER/CHILLS/MYALGIA/JAUNDICE/MUSCULAR

PAIN/SWEATING/ANEMIA/OTHERS)

SIMILAR HISTORY IN FAMILY MEMBERS:

H/O CONTACT WITH PET ANIMALS OR RODENTS:

NATURE OF PET: DURATION OF CONTACT:

SOURCE OF DRINKING WATER:RIVER WATER/POND WATER/ OHT WITH

TAPS/CORPORATION WATER/

RO/PURIFIED WATER

H/O SKIN DISEASE: YES / NO H/O BARE FOOT WALKING IN FARM/ RAIN

WATER/ RODENTS IN INDWELLING

BATHING PLACE: WATER FALLS/RIVER/POND/STAGNANT WATER

SAMPLE	DATE OF COLLECTION	SAMPLE CODE NO	DATE OF PERFORMANCE OF TEST	RESULT	RESULT COMMUNICATED ON

ஒப்புதல் படிவம்.

திரு-திருமதி-செல்வி.----------

----------என்ற

முகவரியில் வசிக்கும் நான் சென்னை மருத்துவக்கல்லூரி மருத்துவமனை மற்றும் ஆராய்ச்சி மையத்தின் நுண்ணுயிர்த்துறை சார்பில் நடத்தப்படும் லெப்டோஸ்பைரோசிஸ் நோய் சம்மந்தமான ஆராய்ச்சிக்கு நான் சம்மதித்து என்னிடம் இரத்தமாதிரி சேகரித்துக்கொள்ள சம்மதிக்கின்றேன்.

இவ்வாராய்ச்சியைப்பற்றி எனக்கு தெளிவாகவும் விளக்கமாகவும் எடுத்துரைக்கப்பட்டது

இவ்வாராய்ச்சிக்கு இம்மருத்துவமனையிலிருந்து பணமோ,பொருளோ ஏதும் பெறவில்லை.

இவ்வாராய்ச்சியின் முடிவுகள் மருத்துவ ஆராய்ச்சிக்கும் மற்றும் மருத்துவக்கல்விக்கும் பயன்படுத்தப்படும் என்பதை அறிந்துகொண்டேன்.

சாட்சி கையெழுத்து.

கையெழுத்து.

பெயர்

பெயர்

ஆராய்ச்சியாளர் கையெழுத்து.

பெயர்

ANNEXURE II

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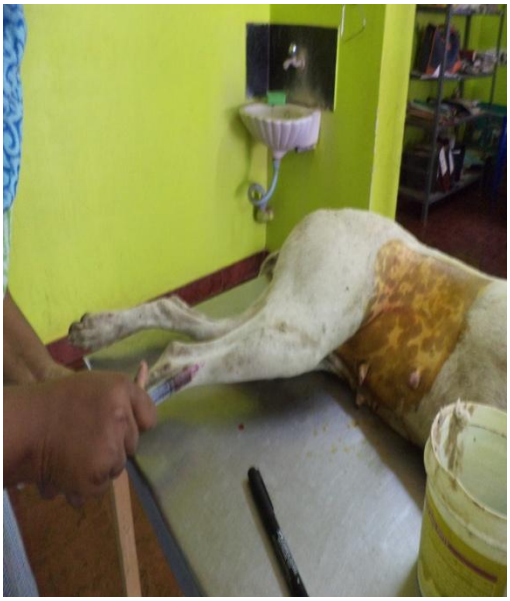
SAMPLE COLLECTION FROM PET DOG



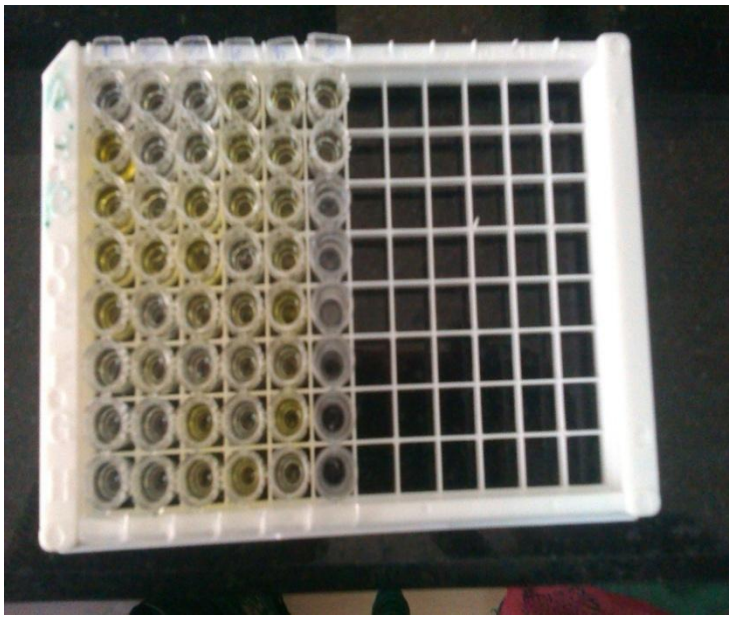
DARK GROUND MICROSCOPE

MEMBRANE FILTER

REFERENCE CULTURES

POSITIVE CULTURES

IgM ELISA MICROTITRE PLATE



CULTURES FOR MAT